GENETIC STUDIES ON FINNISH LUPUS ERYTHEMATOSUS PATIENTS WITH CUTANEOUS MANIFESTATIONS

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To My Loved Ones

Jos sulla kerran on taskussa se suuri unelma,
Niin tämä hetki jää,
Ja vain se mitä teet nyt, on tärkeää.

Don Huonot – Hyvää yötä ja huomenta

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I. Koskenmies S, **Järvinen TM**, Onkamo P, Panelius J, Tuovinen U, Hasan T, Ranki A, Saarialho-Kere U, 2008: Clinical and laboratory characteristics of Finnish lupus erythematosus patients with cutaneous manifestations. *Lupus* 17:337-347.

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V. **Järvinen TM**, Kanninen P, Jeskanen L, Koskenmies S, Panelius J, Hasan T, Ranki A, Saarialho-Kere U, 2007: Matrix metalloproteinases as mediators of tissue injury in different forms of cutaneous lupus erythematosus. *Br J Dermatol* 157:970-980.

* Authors contributed equally. Publication III is included also in the thesis of Anna Hellquist by permission.

AUTHOR CONTRIBUTIONS

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ABBREVIATIONS

ACLE	acute cutaneous lupus erythematosus
ACR	American College of Rheumatology
ANA	antinuclear antibody
BANK1	B-cell scaffold protein with ankyrin repeats 1
BCR	B-cell receptor
BLK	B lymphoid tyrosine kinase
C	complement
CI	confidence interval
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DLE	discoid lupus erythematosus
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
ENA	extractable nuclear antigen
GST	glutathione-S-transferase
GWAS	genome-wide association study
HLA	human leukocyte antigen
HWE	Hardy-Weinberg equilibrium
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
IFN	interferon
IRF5	interferon regulatory factor 5
ITGAM	integrin alpha M
ITGAX	integrin alpha X
LD	linkage disequilibrium
LE	lupus erythematosus
MAF	minor allele frequency
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
OR	odds ratio
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
RA	rheumatoid arthritis
RNA	ribonucleic acid
SCLE	subacute cutaneous lupus erythematosus
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
STAT4	signal transducer and activator of transcription 4
SS	Sjögren's syndrome
Th	T helper cell
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumour necrosis factor-α
TNFAIP3	tumour necrosis factor-α-induced protein 3
TNPO3	transportin 3
TYK2	tyrosine kinase 2
UV	ultraviolet

ABSTRACT

Lupus erythematosus (LE) is a chronic, heterogeneous autoimmune disorder with abnormal immune responses, including production of autoantibodies and immune complexes. Clinical presentations of the disease range from mild cutaneous manifestations to a more generalised systemic involvement of internal organs. Cutaneous (CLE) forms are further subclassified into discoid LE (DLE), subacute cutaneous LE (SCLE) and acute cutaneous lupus erythematosus (ACLE), and may later progress to systemic disease (SLE). Both genetic and environmental factors contribute to the disease, although the precise aetiology is still elusive. Furthermore, complex gene-gene or gene-environment interactions may result in different subphenotypes of lupus. The genetic background of CLE is poorly known and only a few genes are confirmed, while the number of robust genetic associations in SLE exceeds 30.

The aim of this thesis was to characterise the recruited patients (n=310) clinically, and identify genetic variants conferring susceptibility to cutaneous variants of LE. Given that cutaneous and systemic disease may share underlying genetic factors, putative CLE candidate genes for genotyping were selected among those showing strong evidence of association in SLE. The correlation between relevant clinical manifestations and risk genotypes was investigated in order to find specific subphenotype associations. In addition, epistatic interactions in SLE were studied. Finally, the role of tissue degrading matrix metalloproteinases (MMP) in LE tissue injury was explored. These studies were conducted in Finnish case-control and family cohort, and Swedish case-control cohort.

The clinical picture of the patients in terms of cutaneous, haematological and immunological findings resembled that described in the contemporary literature. However, the proportion of daily smokers was very high supporting the role of smoking in disease aetiology. The results confirmed that, even though clinically distinct entities, CLE and SLE share predisposing genetic factors. For the first time it was shown that known SLE susceptibility genes *IRF5* and *TYK2* also increase the risk of CLE. A tendency toward gene-gene interaction between these genes was found in SLE. As a remarkable novel finding, it was observed that *ITGAM* polymorphisms associated even more strongly to DLE than SLE, and the risk estimates were substantially higher than those reported for SLE. Several other recently identified SLE susceptibility genes showed signs of good or modest association especially in DLE. Subphenotype analyses indicated possible associations to clinical features, but marginally significant results reflected lack of sufficient power for these studies. Thorough immunohistochemical analyses of several MMPs demonstrated a role in epidermal changes and dermal tissue remodelling in diseased skin, and suggested that targeted action using selective MMP inhibitors may reduce lupus-induced damage in inflamed tissues.

In conclusion, the results provide an insight into the genetics of CLE and demonstrate that genetic predisposition is at least in part shared between cutaneous and systemic variants of LE. This doctoral study has contributed *IRF5*, *TYK2*, *ITGAM* and several other novel genes to the so far short list of genes implicated in CLE susceptibility. Detailed examination of the

function of these genes in CLE pathogenesis warrants further studies. Furthermore, the results support the need of subphenotype analysis with sample sizes large enough to reveal possible specific disease associations in order to better understand the heterogeneous nature and clinical specificities of the disease. Comprehensive analysis of clinical data suggests that smoking is an environmental triggering factor.

TIIVISTELMÄ

Lupus erythematosus (LE) on monimuotoinen krooninen autoimmuunisairaus, jonka kliininen kuva vaihtelee lievästä ihoon rajoittuvasta diskoidista (DLE) muodosta vakavaan, monia sisäelimiä vahingoittavaan systeemiseen (SLE) muotoon. LE:lle on tyypillistä vasta-aineiden tuotto elimistön omia kudoksia vastaan. Täsmällistä sairastumismekanismia ei tunneta, mutta perintötekijöiden ja ympäristötekijöiden tiedetään vaikuttavan sairastumisalttiuteen. Sairastuneet ovat usein nuoria naisia. Ihomuutokset aiheuttavat sosiaalista haittaa ja potilaiden elämänlaatu on heikentynyt. Parantavaa hoitoa ei ole ja käytetyillä lääkkeillä on haitallisia sivuvaikutuksia.

Iholupukselle altistavien geenien tutkiminen on ollut vähäistä, ja tutkimustulokset ovat ristiriitaisia. Vain muutaman geenin tiedetään varmasti lisäävän riskiä sairastua iholupukseen, kun taas SLE:n alttiusgeenejä tunnetaan yli 30. Lisäksi monimutkaiset geeni-geeni- ja geeni-ympäristövuorovaikutukset saattavat vaikuttaa siihen, sairastuuko potilas iho- vai systeemiseen lupukseen.

Tämän tutkimuksen tarkoituksena oli kuvata kerätyn potilasaineiston ihomuutoksia sekä immunologiaan ja verenkuvaan liittyviä muutoksia ja altistavia ympäristötekijöitä. Tutkimuksessa selvitettiin LE:n ihomuotojen geneettisiä tekijöitä ja mahdollisia geeni-geeni-vuorovaikutuksia. Geneettisiin tutkimuksiin valitut geenit ja niiden polymorfiat olivat sellaisia, joiden tiedetään liittyvän vahvasti SLE:n sairastumisalttiuteen. Lisäksi tutkimme matriksin metalloproteinaasien (MMP) roolia kudosvaurioissa. Tutkimusaineistona käytettiin suomalaisia iholupuspotilaita (n=310) ja terveitä verrokkihenkilöitä (n=356), suomalaisia SLE-perheitä (n=192) ja ruotsalaista tapaus-verrokkiaineistoa (n=164+295).

Suomalaisten potilaiden ihomuutokset sekä immunologiaan ja verenkuvaan liittyvät löydökset olivat samanlaisia kuin kirjallisuudessa aikaisemmin kuvatuilla potilailla. Huomattavan suuri osa potilaista tupakoi ensimmäisten oireiden ilmetessä. Geneettisissä tutkimuksissa osoitimme, että *IRF5*- ja *TYK2*-geenit lisäävät riskiä sairastua iholupukseen. Havaitsimme myös tilastollisesti merkitsevän interaktion näiden kahden geenin välillä SLE-potilaissa. Terveisiin verrokkihenkilöihin verrattuna *ITGAM*-geenin polymorfiat lisäsivät kolminkertaisesti riskiä sairastua DLE:hen. Myös muut hiljattain tunnistetut SLE:n alttiusgeenit assosioituivat iholupukseen. Kliinisten alatyyppien ja tutkittujen geenipolymorfioiden välillä havaittiin vain heikkoja assosiaatioita, mikä johtunee liian pienestä otoskoosta. Tutkimustulokset osoittivat, että lupuksen eri alatyyppien MMP-ilmentymisprofiili on hyvin samankaltainen. Tämän perusteella voidaan ehdottaa, että MMP-estäjien kohdennettu käyttö saattaa vähentää lupuksesta johtuvia kudosvaurioita tulehdusalueilla.

Tässä tutkimuksessa saadut tulokset lisäävät tietoutta iholupuksen geneettisestä taustasta ja osoittavat, että lupuksen eri muodoilla on ainakin osittain yhteinen geneettinen pohja. Uutena tuloksena olemme osoittaneet, että *IRF5*-, *TYK2*-, *ITGAM*- ja monet muut geenit eivät

assosioidu vain SLE:hen vaan myös iholupukseen. Näiden geenien vaikutusmekanismit iholupuksen synnyssä ovat toistaiseksi tuntemattomia. Sairauden monimuotoisuuden ymmärtämiseksi on tärkeää analysoida kliinisten erityispiirteiden ja geneettisten tekijöiden välistä yhteyttä. Riittävän iso otoskoko on kuitenkin edellytys luotettavien tulosten saamiseksi. Tupakointia voidaan pitää mahdollisena altistavana ympäristötekijänä, joka voi lisätä sairastumisriskiä geneettisesti alttiissa henkilöissä.

INTRODUCTION

Lupus erythematosus (LE) is a heterogeneous autoimmune disease with a varying clinical course. Manifestations range from skin-restricted lesions to a multi-organ, systemic disease. From a clinical standpoint the disease is subdivided into discoid (DLE), subacute cutaneous (SCLE) and systemic (SLE) forms, all of which have their characteristic immunological and haematological stigmata. LE is a multifactorial disease, as both genetic and environmental factors contribute to disease onset. The molecular level pathogenesis is, however, poorly understood.

Comprehensive descriptive surveys conducted in cutaneous lupus (CLE) patients are sparse and often confounded by patient misclassification. Furthermore, genetic ancestry may affect the presence of clinical manifestations. This emphasises the need to carefully verify recruited patients both clinically and genetically. A strong genetic component similar to that of SLE may also underlie CLE. There are a few confirmed predisposing genes for DLE and SCLE including *HLA*, *TNF-α* and complement genes. Many genes outside the *HLA* region have shown signs of linkage or association, but their role needs to be confirmed. The genetic background of SLE has been extensively studied and over 30 genes have reached significant association. Many of these genes fall in key pathways implicated in disease pathogenesis. Epistasis, in other words, gene-gene interaction has been recognised for long but it has not been specifically addressed in LE. Subphenotype analysis may shed light on the overwhelming clinical variability of the disease, but attempts to perform these analyses have been unsuccessful, probably due to limited sample size and low statistical power.

The central aim of this study was to attain a well-characterised group of CLE patients as the basis for genetic studies. For this purpose, over 300 patients with DLE, SCLE or SLE were recruited and evaluated. Clustering of autoimmune diseases in families and shared pathogenic features between CLE and SLE implicate that there may be common genes underlying disease progression. Therefore, we set out to genotype a group of genes previously implicated in SLE for their association with CLE.

REVIEW OF THE LITERATURE

1 Structure of the skin

The skin is the largest and most complex organ of the human body and provides a protective interface between the organism and pathogens invading from the environment (Rao *et al.* 1996). Skin consists of two compartments, namely the outer epidermis and the inner dermis (Vitellaro-Zuccarello 1997). The principle cells in the epidermis are keratinocytes that differentiate and migrate progressively through epidermal layers. These layers include the innermost proliferating basal layer (*stratum basale*); the spinous layer (*stratum spinosum*); the granular layer (*stratum granulosum*); and the outermost horny layer (*stratum corneum*). Three other types of cells are also present in the epidermis: antigen-presenting Langerhans cells, pigment-producing melanocytes and Merkel cells, which are mechanoreceptors. Beneath the epidermis lies the dermis, a dense connective tissue, where blood vessels are also located.

2 Historical aspects of cutaneous lupus erythematosus

The term *lupus érythémateaux* was introduced for the first time in 1851 (Rowell 1997). The Latin word *lupus* means wolf and *erythema* is defined as redness of the skin occurring with skin injury, infection or inflammation. Another symbol commonly used in the context of the disease is a *butterfly*. These words are apt in describing the disorder in which patients may have gnawed facial injuries resembling a wolf bite or a butterfly-shaped rash extending over the face. The term lupus appeared in medical literature over 1000 years ago (Rowell 1997). An early description of the discoid form of cutaneous lupus dates back to 1833, although the word discoid was not coined until 40 years later and eventually the concept was refined according to the distribution and extent of the skin changes in 1934 (Rowell 1997). Apparently the first delineation of subacute cutaneous disease was already published at the end of 19th century (Rowell 1997), even though the case report by Richard D Sontheimer in 1979 (Sontheimer *et al.* 1979) is generally considered a fundamental description. Some patients, however, have fever, anaemia and pain around joints in conjunction with cutaneous manifestations, and these symptoms were named as systemic in 1875. By the early part of the next century systemic lupus had become recognised, while the discovery of autoantibodies and antinuclear antigens in 1950-1960's (Meller *et al.* 2005) improved the understanding of the disease with autoimmunity in nature and high variability.

3 Classification and nomenclature of lupus erythematosus

Lupus erythematosus (LE) is a heterogeneous autoimmune disease associated with aberrant immune responses including production of autoantibodies and immune complexes. Clinical presentations of the disease range from mainly skin-confined manifestations to a progressive multisystem disease with involvement of internal organs, such as kidney, lung, heart and central nervous system. Individual disease susceptibility is a complex puzzle of multiple

genes, gender and environmental triggering factors, making the identification of these pieces a challenging task.

3.1 Cutaneous lupus erythematosus

Nomenclature for the cutaneous manifestations of LE-related skin disease was initially proposed by James N Gilliam in 1977 (Sontheimer 1997). This classification scheme divides all skin lesions into LE-specific and LE-nonspecific skin disease (Table 1). LE-specific skin disease includes the classic subtypes of cutaneous lupus erythematosus (CLE) with distinct histopathological features: chronic cutaneous lupus erythematosus, of which discoid lupus erythematosus (DLE) is the most common form, subacute cutaneous lupus erythematosus (SCLE) and acute cutaneous lupus erythematosus (ACLE). Histologically nonspecific LE lesions are associated with LE, but are not specific to the disease process itself. That is to say, identical lesions can also be encountered in other disease processes. Nonspecific skin lesions, such as vasculitis, livedo reticularis and alopecia are seen in patients with CLE (Tebbe 2004); in SLE these lesions are more frequent and reflect disease activity (Werth 2005).

Gilliam meant his nomenclature to be a logical classification system for the extremely varied skin changes rather than a rigid scheme to define subsets of LE (Sontheimer 1997). The nebulous usage of terminology over the years has resulted in several attempts to provide new sets of criteria, however, with no satisfactory approach being addressed (Sontheimer 1997).

Table 1. The Gilliam classification of skin lesions associated with LE (modified after Lin *et al.* 2007).

LE-specific skin disease	**LE-nonspecific skin disease**
Chronic cutaneous LE	Cutaneous vascular disease
1. Classic discoid LE	Nonscarring alopecia
a. Localised DLE	Sclerodactyly
b. Generalised DLE	Rheumatoid nodules
Subacute cutaneous LE	Calcinotis cutis
1. Annular SCLE	LE-nonspecific bullous lesions
2. Papulosquamous/psoriasiform	Urticaria
Acute cutaneous LE	Papulonodular mucinosis
1. Localised (malar rash; butterfly rash)	Cutis laxa/anetoderma
2. Generalised acute cutaneous LE (SLE rash)	Acanthosis nigricans
	Erythema multiforme
	Leg ulcers
	Lichen planus

3.2 Systemic lupus erythematosus

The classification of systemic lupus erythematosus (SLE), which is a multisystem disease characterised by the production of several autoantibodies, is based on the criteria established by the American College of Rheumatology (ACR) (Hochberg 1997; Tan *et al.* 1982). The fulfilment of four out of 11 clinical and laboratory criteria (Table 2) is sufficient for verification of SLE. The current criteria have been criticised for emphasising skin too much in the identification of a multiorgan disease (Albrecht *et al.* 2004). Four dermatologic features are included, namely butterfly rash, discoid lupus, photosensitivity and oral ulcers. Consequently, individuals presenting with only the aforementioned skin manifestations can be classified as having SLE. On the other hand, a maximum of 20% of DLE and up to 50% of SCLE patients have four or more positive ACR criteria (Kuhn and Sontheimer 2008; Sontheimer 2005). Thus it is evident that the ACR criteria characterise a heterogeneous group of patients and are less useful for exclusion of SLE in patients with predominant cutaneous manifestations (Kuhn and Sontheimer 2008). Reevaluation and recommendations have been made and suggest that the revised criteria should include Ro/SSA and La/SSB autoantibody testing, skin biopsy and control groups representing relevant differential diagnoses in sufficient numbers to assess specific diagnostic problems (Albrecht *et al.* 2004).

Table 2. The American College of Rheumatology Criteria for the classification of SLE (modified from Wakeland *et al.* 2001).

Category	**Symptom**
Skin criteria	Butterfly rash
	Discoid rash
	Photosensitivity
	Oral ulcerations
Systemic criteria	Arthritis
	Serositis (pleuritis or pericarditis)
	Proteinuria
	Neurological disorders (seizures or psychosis)
Laboratory criteria	*Haematologic:*
	Haemolytic anaemia, leukopaenia, lymphopaenia or thrombocytopaenia
	Immunologic:
	Antibodies against dsDNA, Sm and phospholipids, such as cardiolipin
	Antinuclear antibodies (ANAs)

3.3 Diagnosis

The diagnosis of different subtypes of LE is based on clinical and histopathological features in combination with laboratory abnormalities and immunogenetic patterns specific for, but not limited to, a subtype. Skin biopsy showing LE-specific histology is needed to confirm the diagnosis (Werth 2005), but is less useful in determining the clinical subtype (Walling and Sontheimer 2009). Assessment of the autoantibody profile is helpful in contributing to the presence of SLE, but has a more limited role in the diagnosis of CLE (Walling and Sontheimer 2009). Signs and symptoms evolve over time and therefore, it may take several years before the proper diagnosis is made; for SLE an average of four years is spent before a correct diagnosis is reached (Manzi 2009). On the other hand, SLE is often over-diagnosed. Only about half of the individuals with presumptive diagnosis by a nonrheumatologist could be confirmed as having SLE (Narain *et al.* 2004).

As skin disease is the second most frequent clinical manifestation of SLE (Lin *et al.* 2007; Werth 2005), all patients presenting with cutaneous findings should be comprehensively evaluated for possible systemic involvement at baseline (Walling and Sontheimer 2009). In addition, certain patterns of systemic disease activity can be seen in cutaneous forms of LE. Therefore, it is important to carefully follow up these patients to assure that SLE does not develop. For instance, patients with SCLE are reported to have mild systemic illness marked with musculoskeletal complaints and serological abnormalities at the time of cutaneous outbreak (Sontheimer 2005). Severe central nervous system or renal defects are rare. Approximately one third of DLE patients have extracutaneous signs of the disease (Kuhn and Sontheimer 2008). It has been estimated that 5% of patients with localised and 20% with generalised DLE lesions develop SLE, whereas about 10-15% of SCLE cases progress to an overt systemic disease (Lee and Sinha 2006; Sontheimer 2005). Variables important for disease progression to SLE include signs of nephropathy, arthralgias and elevated antinuclear antibody (ANA) titres (≥1:320) (Tebbe *et al.* 1997). It has been suggested that high levels of Ro/SSA autoantibodies in SCLE patients may serve as a prognostic tool to predict development of systemic inflammatory disease (Popovic *et al.* 2007a).

CLE is not a life-threatening illness and has an excellent prognosis. Nevertheless, scarring lesions can be socially debilitating and patients often experience reduced quality of life (see section 7). Lethal outcome seems to be a rare event; in a 10 year follow-up 10% of the SCLE patients died, but only one from LE complications (Tebbe and Orfanos 1997). The survival of patients with SLE has improved markedly over the past 50 years, and today up to 90% of patients survive at least ten years after diagnosis of SLE (Manzi 2009). Unfortunately, prognosis of SLE remains considerably poorer in many developing countries (Borchers *et al.* 2009). Despite better outcome, patients are now facing a greater risk of diseases related to ageing, such as cardiovascular disease, cancer and osteoporosis (Manzi 2009). Among patients with SLE, the incidence ratio for cancer is 1.2; the risk of myocardial infarction is six times higher in female SLE patients aged 24 years compared to control individuals with similar age.

4 Clinical and histopathological features

4.1 Discoid lupus erythematosus

Women are affected at the 2-3:1 ratio with a typical onset age of 30 years. A rash is usually the sole manifestation of DLE and well-demarcated, erythematous, scarring plaques, being the most common skin involvement, are seen in the head and neck region, although palms and soles can be affected as well. (Crowson and Magro 2001). Lesions on the head and neck region are referred to as localised whereas those occurring both above and below the neck are called generalised DLE (Lee and Sinha 2006). Occasional patients with disseminated lesions will manifest haematological and other abnormalities indicative of supervening SLE. Skin without lesions rarely contains deposits of immunoglobulins that are seen as a band-like structure in biopsy specimens obtained from patients with DLE (see section 4.4). (Crowson and Magro 2001).

4.2 Subacute cutaneous lupus erythematosus

The disease incidence is 3-4 times higher in women than in men and patients are typically in their 20s and 30s (Lee and Sinha 2006). Patients present with papulosquamous, psoriasiform and/or annular-polycyclic skin eruption on the upper trunk and experience enhanced photosentivity (Crowson and Magro 2001). SCLE lesions typically resolve without scarring. Approximately one fifth of patients have been reported to develop typical DLE or ACLE lesions during their disease course. (Sontheimer 2005). Affected individuals may also have a wide range of other LE-specific (such as mouth ulcers) or LE-nonspecific (such as vitiligo-like lesions and nonscarring alopecia) skin manifestations (Crowson and Magro 2001). High titres of Ro/SSA autoantibodies against extractable nuclear antigens (ENA) are frequently displayed in the sera obtained from patients (Sontheimer 2005). SCLE may be induced by a variety of medications, such as antihypertensive drugs, especially calcium channel blockers (Sontheimer *et al.* 2009; Srivastava *et al.* 2003).

4.3 Acute cutaneous and systemic lupus erythematosus

The peak of onset is around 30 years of age, although manifestations may appear at any time point from early to mid adulthood. There is a female predominance: nine out of ten affected individuals are women. Articular, that is, joint-related defects are common at the outset, but ultimately 80% of the SLE patients will have cutaneous manifestations during the course of their disease (Crowson and Magro 2001). A localised malar, butterfly-shaped rash over the face and bridge of the nose is most commonly seen and may occur concurrently with other symptoms of lupus or precede systemic illness by months to years (Lee and Sinha 2006). ACLE typically presents abruptly in the context of systemic disease; the clinical activity of the lesions cycles in parallel with the activity of underlying SLE (Lee and Sinha 2006; Rothfield *et al.* 2006). The appearance of generalised lesions in the form of erythema of the face, upper trunk or extremities is usually related to sun exposure, to which ACLE is frequently associated (Rothfield *et al.* 2006). Dissemination of the lesions usually coincides

with exacerbation of systemic manifestations (Kuhn and Sontheimer 2008). Over 70% of ACLE patients meet the ACR criteria for SLE that is utilised for verifying the diagnosis together with vesicles and bullae arising on sun-exposed skin (Werth 2005). Virtually all patients with ACLE eventually progress to overt SLE (Lee and Sinha 2006). Although the different forms of CLE are usually thought of as distinct entities of LE, any of their dermatological changes can occur in patients with SLE (Yell *et al.* 1996). A minority of patients (15-20%) have skin involvement resembling that of DLE and lesions mimicking the annular shape seen in SCLE are found in a few patients (10-15%) positive for Ro/SSA autoantibodies. ACLE lesions are typically seen in 30-50% of SLE patients.

4.4 Characteristic histopathology

Typical histopathological skin changes include interface dermatitis accompanied by lymphocytic infiltrate which is most prominent in DLE; hyperkeratosis; epidermal atrophy, i.e. thinning of the epithelium especially in SCLE; vacuolisation of the basal epidermis is often seen in SLE; epidermal basement membrane thickening; and melanin pigment incontinence (Crowson and Magro 2001; Rothfield *et al.* 2006). Varying combinations of these features are seen in CLE and SLE. Conventional histopathology is reinforced with immunopathological lupus band testing that is often referred to as DIF, direct immunofluorescence testing. The lupus band is defined as deposits of immunoglobulins and complement proteins along the dermal-epidermal junction of lesional skin biopsy specimens. More than 60% of patients with CLE have proteins in their lesional skin. Therefore, positive test result supports the diagnosis of LE, but a negative finding does not rule out a diagnosis of lupus. (Rothfield *et al.* 2006).

5 Laboratory findings

5.1 Haematological features

Systemic disease is accompanied with various haematological abnormalities that are rarely seen in CLE (Wallace *et al.* 1992). These include anaemia, leukopaenia, lymphopaenia and thrombocytopaenia. In other words, patients have insufficient haemoglobin levels and reduced numbers of red blood cells, leukocytes, lymphocytes and platelets. In addition, sedimentation rate, a measure of inflammation and immune activation, is elevated in almost all patients and falls to normal levels during the inactive phase of the disease. Serum complement (C) levels, the most important being C3 and C4, are also depressed. (Rothfield *et al.* 2006).

5.2 Autoantibodies

SLE is virtually always accompanied by the production of autoantibodies of multiple specificities (Arbuckle *et al.* 2003), which are infrequent in DLE (Wallace *et al.* 1992). Some autoantibodies may contribute directly to the pathogenic changes of SLE (Arbuckle *et al.* 2003), while their role in CLE is less clear. Autoantibodies precede the diagnosis of SLE by

many years and their appearance tends to follow a predictable course (Arbuckle *et al.* 2003). Virtually all patients with SLE have a positive ANA test (Rothfield *et al.* 2006). However, ANAs are by no means specific for SLE; they can be detected in 25-80% of patients with CLE (Tebbe 2004) and sporadically in 2% of the female population over the age of 40 (Wakeland *et al.* 2001). Instead, antibodies against double-stranded deoxyribonucleic acid (dsDNA), Smith (Sm) antigens and ribonucleoprotein (RNP) are fairly specific for SLE, while less common in CLE (Chlebus *et al.* 1998; Rothfield *et al.* 2006; Sontheimer 2005). Ro/SSA autoantibodies are considered characteristic for SCLE, occurring in approximately 70% of patients (Sontheimer 2005). These autoantibodies are seen in SLE with similar frequency than in SCLE (Chlebus *et al.* 1998), whereas the majority of DLE patients are negative for Ro autoantibodies (Lee *et al.* 1994; Popovic *et al.* 2007a). A minority of the healthy population presents with Ro autoantibodies as well, implicating nonspecificity for LE (McCauliffe 1997). Subjects positive for Ro tend to have cutaneous manifestations and photosensitivity not necessarily associated with LE or Sjögren's syndrome (SS) (McCauliffe 1997). Follow-up studies have shown that Ro-positive patients with diverse clinical presentation at baseline have a dynamic disease process with skin disease progression and development of new autoimmune diseases (Popovic *et al.* 2008; Simmons-O'Brien *et al.* 1995). The typical clinical and histopathological features as well as laboratory findings of DLE, SCLE and SLE are summarised in Table 3.

Table 3. Characteristic clinical, histopathological, haematological and immunological features for DLE, SCLE and SLE.

	DLE	**SCLE**	**ACLE/SLE**
Age at diagnosis	30 y	20-30 y	30 y
Female to male ratio	2-4:1	3-5:1	9:1
Type of skin manifestation	Well-demarcated, erythematous, scarring	Papulosquamous or annular	Malar, butterfly-shaped
Location of lesions	Head and neck	Upper trunk	Over the face
Charateristic histopathology	Interface dermatitis, hyperkeratosis ±, follicular plugging ±, perivascular and periadnexal lymphocyte inflammation	Interface dermatitis, epidermal atrophy	Interface dermatitis
Positive lupus band test on lesional skin	90%	60%	>90%
Photosensitivity	42%	64%	25%
Extracutaneous involvement	−	Joints	Kidney, lung, heart, central nervous system
Haematological changes	+	++	+++
Autoantibodies	ANA ± ENA ± dsDNA −	Ro/SSA + La/SSB + dsDNA ±	ANA + dsDNA + Sm +

6 Other clinical manifestations

Besides the affected skin, general symptoms, such as fever and fatigue, are present in a high proportion of SLE patients. Fatigue can be seen during periods of disease activity and is associated with pain, insomnia and depression (Walling and Sontheimer 2009). More than 80% of patients have low grade or spiking fever at the time of diagnosis (Rothfield *et al.* 2006). Furthermore, patients with SLE suffer from extensive visceral involvement (Rothfield *et al.* 2006). This includes cardiovascular manifestations and renal disease as well as central nervous system involvement in forms of severe headaches, seizures and organic brain damage. Depression and anxiety are common psychological problems. Fever, headache, cardiovascular disease and arthritis/arthralgias are most often recorded for patients with CLE, while renal involment is virtually absent (Chlebus *et al.* 1998; Popovic *et al.* 2007b; Wallace *et al.* 1992). Symptoms of the nervous system tend to be much milder in patients with SCLE than in SLE (Walling and Sontheimer 2009). Other autoimmune diseases, such as SS, may be present in both SCLE and SLE (Rothfield *et al.* 2006; Sontheimer 2005); the prevalence of SS is 43% in SCLE (Black *et al.* 2002) and 20% in SLE (Ramos-Casals *et al.* 2007).

7 Quality of life

The physical, psychological as well as economic burden of the disease is especially devastating because young adults are often affected. As a potentially disabling disease persisting over decades and not infrequently associated with vocational handicap and unemployment, LE may lead to limited quality of life (Meller *et al.* 2005). The effects of skin disease on patients' lives are measured with the self-administrated Skindex questionnaire that consists of 29 items related to daily performance, emotions and symptoms on a 5-point response scale (Chren *et al.* 1997). Patients with widespread DLE experienced the greatest worsening of life quality (mean Skindex score=93.6), the impact being somewhat smaller in SCLE (79.4), ACLE (76.3) and localised DLE (74.2) (Moghadam-Kia *et al.* 2009). The mean CLASI damage score was also highest among subjects with DLE (mean score=10.2). These results are not surprising as DLE lesions typically produce marked facial scarring and dyspigmentation as they resolve and nearly 45% of DLE patients have a vocational handicap (Moghadam-Kia *et al.* 2009). Furthermore, CLE is considered to be the third most common cause of industrial disability from dermatological diseases (Tebbe and Orfanos 1997).

8 Treatment for cutaneous lupus erythematosus

General treatment for CLE includes photoprotective strategies: avoidance of sunlight, artificial sources of UV exposure and photosensitising drugs, as well as use of broad spectrum sunscreens (Kuhn *et al.* 2005). Smoking cessation should be encouraged, as evidence indicates that smoking interferes with the efficacy of antimalarial therapy and exacerbates disease activity (Walling and Sontheimer 2009). Because of their favourable safety profile, topical therapies, such as corticosteroids and calcineurin inhibitors are most often employed (Sontheimer 2005; Walling and Sontheimer 2009). Widespread skin manifestations are treated with oral antimalarial drugs (chloroquine, hydroxychloroquine) (Kuhn *et al.* 2005). For patients with resistant disease immunosuppressive agents (methotrexate and azathioprine)

or thalidomide are arguably the most efficacious (Kuhn *et al.* 2005; Rothfield *et al.* 2006; Walling and Sontheimer 2009). Recombinant tumour necrosis factor (TNF)-α inhibiting agents may turn out to be a successful biologic therapy. However, their ability to induce dermatological changes dampens promising views (Rothfield *et al.* 2006; Walling and Sontheimer 2009).

9 Prevalence and incidence

Prevalence and incidence are used as estimates of how common a condition is within a population at a given time. Prevalence is defined as the total number of cases, and incidence as the number of new cases within a time frame, usually a year (Danchenko *et al.* 2006). Although population-based studies assessing the prevalence of CLE are still lacking, it is estimated to be two to three-fold more prevalent than SLE (Tebbe and Orfanos 1997) and prevalence is dependent on the population studied (Lin *et al.* 2007). The prevalence of SLE in Finland is 28/100,000 (Helve 1985). Worldwide SLE rates show considerable disparities, prevalence being the lowest in Northern Ireland (25/100,000) and the UK (26/100,000) and the highest in Spain (91/100,000). Incidence was found to be the lowest in Iceland (3/100,000) and Japan (3/100,000) and the highest in the USA (5/100,000) and France (5/100,000). This high variability may result from methodological differences of studies or reflect true differences across populations. (Danchenko *et al.* 2006).

At rheumatology practice, one CLE case for every seven cases of SLE has been reported (Wallace *et al.* 1992). Fresh figures from the USA show a prevalence of 73/100,000 and an incidence of 4/100,000 for CLE (Durosaro *et al.* 2009). One population-based study suggested a prevalence of 28/100,000 for DLE, while the incidence (2/100,000) was estimated to be quite similar for DLE and SLE (reviewed in Lin *et al.* 2007). More recently a Swedish study estimated that the prevalence of Ro/SSA-positive SCLE is 6-14/100,000 and incidence is 0.7/100,000 (Popovic *et al.* 2007b). Furthermore, some ethnic differences in prevalence have been reported: SCLE is uncommon in African-Americans, Koreans and Chinese, while DLE is more prevalent in African-Americans than Caucasians (Sontheimer 2005). SLE is far less common in Europeans and their descendants compared to other ethnicities (Borchers *et al.* 2009).

10 Aetiology

The aetiology of LE is not still fully understood, but multiple factors such as genetic predisposition, environmental exposure and hormonal factors contribute (Criswell 2008). Autoimmunity develops in many cases over years by a process that combines immune activation, genetic susceptibility and environmental factors in a certain individual at a certain time point (Nancy and Yehuda 2009). Based on this, a tentative model has been proposed (Rhodes and Vyse 2007; Rhodes and Vyse 2008; Wandstrat and Wakeland 2001). In this scenario, individuals have a genetic predisposition (see sections 14.1 and 14.2) to disease according to the number of susceptibility alleles they carry; genetic susceptibility, on the other hand, is determined at birth. Limited heritability (Lawrence *et al.* 1987) and onset at

adulthood imply that a number of triggers occurring together or sequentially over a limited period of time are required for the disease to develop, which happens when a threshold of genetic and environmental susceptibility effects is reached. For those with little genetic risk, disease may never develop despite strong or prolonged exposure to the relevant environmental triggers.

10.1 The female gender

In general, women are more prone than men to develop autoimmune diseases. The female preponderance is not seen only in LE, but also in SS (approximately 90% of patients are women), RA (>70%) and multiple sclerosis (>65%) (Invernizzi *et al.* 2009). Estimations on the female to male ratio vary in CLE (2-3:1), but it seems that the female predominance is much less pronounced than in SLE (9:1) (Tebbe and Orfanos 1997; Vera-Recabarren *et al.* 2009b). A large cohort study from the 1970s reported equal numbers of women and men with DLE (Lin *et al.* 2007), while more recent studies observed a female to male ratio of 4:1 for DLE (Moghadam-Kia *et al.* 2009) and that of 3-5:1 for SCLE (Moghadam-Kia *et al.* 2009; Popovic *et al.* 2007a). Reported gender differences in disease severity and manifestations are few. According to one study that investigated 205 female and 103 male patients with CLE, affected women had a higher prevalence of clinical and immunological abnormalities (Vera-Recabarren *et al.* 2009b). For instance, women had more often arthralgias (29%) and elevated ANA titres (46%) compared to men (12% and 17%, respectively). Though less common in men, when it does occur, SLE tends to run a more severe course (Yacoub Wasef 2004).

The reasons for the gender bias in autoimmune diseases remain elusive, although sex hormones may play a role. Female hormones promote humoral immune responses and lymphocyte self-reactivity (Ackerman 2006). An alternative theory is related to the gene-dose effect of genes located on the X chromosome (Invernizzi *et al.* 2009) and epigenetic mechanisms regarding altered methylation patterns of genes in the inactive X chromosome in women (Hewagama and Richardson 2009). The risk of SLE in men with Klinefelter's syndrome (47, XXY) is predicted to be 14 times higher compared with normal men (46, XY) (Scofield *et al.* 2008). In addition, the SLE candidate gene *MEC2P* that regulates methylation is located on the X chromosome (Rhodes and Vyse 2008; Suarez-Gestal *et al.* 2009). The role of sex hormones in disease pathogenesis is undeniable, but a more complex interplay of hormones and epigenetic mechanisms, like methylation, cannot be excluded.

10.2 Environmental triggering factors

The nature of environmental triggers predisposing to LE is partly unknown, but some of them may already operate early in life (Edwards and Cooper 2006). Irrespective of the environmental exposure, it is most likely a common factor of low penetrance, as otherwise there would be a dramatic increase of cases among individuals with the relevant exposure. One might also hypothesise that it is unlikely to be a single common factor because LE is not a highly prevalent disease. (Rhodes and Vyse 2008). Numerous environmental factors are implicated in LE, and autoimmunity in general including ultraviolet (UV) light, cigarette

smoking, medications, infectious agents, chemicals and diet (Nancy and Yehuda 2009; Sarzi-Puttini *et al.* 2005; Shapiro *et al.* 2004). Common to these aforementioned precipitants is that they induce apoptosis, autoantigen translocation to cell surface and other inflammatory events known to trigger LE (Shapiro *et al.* 2004).

10.2.1 Smoking

Several studies have consistently showed an association between cigarette smoking and CLE (Boeckler *et al.* 2009; Gallego *et al.* 1999; Lipsker *et al.* 2006; Miot *et al.* 2005) and reported up to a ten-fold excess risk for the development of DLE (Gallego *et al.* 1999; Miot *et al.* 2005). Notably, most of the refractory cases not responding to treatment were currently smoking female DLE patients (Moghadam-Kia *et al.* 2009). Current, rather than past, smoking may be a hazard for the development of SLE (Costenbader *et al.* 2004), but the relationship is somewhat controversial (Costenbader and Karlson 2005) and the immunologic subphenotype of an individual appears to play a role (Freemer *et al.* 2006). Smoking is significantly associated with numerous adverse cutaneous consequences (Freiman *et al.* 2004). In addition, tobacco smoke contains toxic components that have multiple biological effects from tissue damage to immune system modulation (Costenbader and Karlson 2006; Nancy and Yehuda 2009). However, little is known about the exact pathological mechanisms involved (Boeckler *et al.* 2009). The challenge will be to convince patients to cease smoking since it clearly poses increased risk and, on some occasions, cessation of smoking has resulted in complete remission of LE (Lipsker *et al.* 2006; Miot *et al.* 2005). Meanwhile, the underlying mechanism and gene-environment interactions in genetically prone individuals deserve to be studied in more detail.

10.3 Ultraviolet light

Exposure to UV light is one of the major factors known to trigger cutaneous disease activity. Sunlight can precipitate disease *de novo* or aggravate existing disease. Summertime exposure to UV light may lead to systemic flares three to six months later (Lin *et al.* 2007). The different wavelengths of UV spectrum, namely UVA and UVB have varying biological effects on the skin (Angotti 2004; Lin *et al.* 2007). UVB does not penetrate deeper than the epidermal layer and is strongly absorbed by DNA, while UVA penetrates deeper into the dermis (Angotti 2004). Potential molecular targets include DNA, ribonucleic acid (RNA), proteins and lipids (Angotti 2004; Lin *et al.* 2007). UVB has significant photobiological effects including induction of apoptosis through DNA damage; translocation of nuclear autoantigens to the surface of keratinocytes, thereby stimulating an autoimmune response; generation of novel antigenic products; and dermal vessel activation (Angotti 2004; Kuhn and Bijl 2008). Cutaneous inflammation is further promoted by UVB-induced release of proinflammatory cytokines, such as interleukin (IL)-10 and TNF-α, which in turn induce adhesion molecule and chemokine expression to facilitate inflammatory cell, as well as interferon-producing cell, recruitment into the skin (Angotti 2004; Lin *et al.* 2007). Consequently, more effector cytokines are released, thus amplifying chemokine production and leukocyte recruitment (Lin *et al.* 2007).

10.3.1 Photosensitivity

Three of the 11 ACR classification criteria, namely malar rash, discoid LE and photosensitivity, relate to photodistribution, suggesting an important role of UV light in the pathogenesis (Lin *et al.* 2007). Sensitivity to light in forms of prolonged and delayed erythema is a common feature in all patients with cutaneous or systemic disease (Lehmann and Homey 2009). The strong association between photosensitivity and LE has led to the assumption that abnormal photoreactivity is pivotal for the pathogenesis of cutaneous and systemic variants of the disease (Kuhn *et al.* 2005). However, the ACR definition of photosensitivity is extremely vague and fulfilled when patient or treating physician reports an adverse reaction to sunlight, and may encompass a number of related dermatoses, such as polymorphous light eruption (Lin *et al.* 2007). Malar rash is often indistinguishable from photosensitivity and these two criteria were criticised as not being independent in the classification of SLE (Albrecht *et al.* 2004). A detailed clinical history of patients including morphology and distribution of the rash, its duration and relation to sun exposure is vital for the diagnosis and assessment of photosensitivity, although negative history does not necessarily rule out sensitivity to sun light (Kuhn and Bijl 2008). For some patients it might be difficult to link the worsening of their disease to the detrimental effects of sunlight because of the latency period of up to several weeks in the development of skin lesions (Lehmann and Homey 2009). Therefore, a standardised photoprovocation test performed at different wavelengths has been developed to evaluate photosensitivity (Lehmann and Homey 2009), as both single and repeated exposures to UV radiation provoke skin lesions in patients (Angotti 2004). Pathologic skin reactions are most pronounced in SCLE followed by DLE and SLE (Lehmann *et al.* 1990). Not all patients, however, show positive reaction after phototesting despite their history of photosensitivity (Lehmann and Homey 2009), which may be due to delayed lesion formation, interindividual variation, methodological differences and bias in patient selection (Kuhn and Bijl 2008; Lehmann and Homey 2009). A practical consequence of photosensitivity is that patients are not adequately protected by window glass or by conventional sunscreens (Kuhn and Sontheimer 2008).

10.4 Apoptosis

A direct effect of sun exposure is the induction of apoptosis in suprabasal keratinocytes (Angotti 2004). Programmed cell death or apoptosis is a normal biological process characterised by cell shrinkage, nuclear condensation, cytoplasmic contraction and the packaging of cellular components within membranes before their budding from the apoptotic cells as apoptotic bodies. UV light has the capability to induce apoptosis in keratinocytes with different mechanisms, but the complex molecular machinery controlling keratinocyte apoptosis in the epidermis is still poorly known (Angotti 2004). In normal physiological conditions, apoptotic keratinocytes are eliminated quickly to prevent the release of harmful intracellular constituents and their exposure to the immune system (Bijl and Kallenberg 2006). In CLE skin, expression of apoptosis blocking protein, Bcl-2, is diminished, and accordingly, the number of apoptotic keratinocytes is increased (Baima and Sticherling 2001; Chung *et al.* 1998; Norris *et al.* 1997; Pablos *et al.* 1999). This increase correlates with the

upregulation of p53 (Chung *et al.* 1998; Pablos *et al.* 1999) by UV-induced DNA damage and/or TNF-α (Lin *et al.* 2007). Therefore, the increased rate of apoptosis could be mediated directly by UV light or be a consequence of UV-induced cytokine mechanisms (Angotti 2004). It was further demonstrated that apoptotic cells tend to accumulate in the skin of patients after UV radiation possibly as a result of impaired or delayed clearance (Kuhn *et al.* 2006). Unremoved cells may release proinflammatory compounds and potential autoantigens, and consequently provoke tissue inflammation and an autoimmune response. A similar increase in apoptosis rate, clearance defects and consecutive accumulation of apoptotic cells has been reported to occur also in SLE (Baumann *et al.* 2002; Emlen *et al.* 1994; Gaipl *et al.* 2006; Lorenz *et al.* 2002). Impaired clearance by phagocytosis may be partly genetically determined (Mok and Lau 2003). A novel theory anticipates a role for Ro52 autoantibodies which are upregulated in CLE keratinocytes in response to UV light (Oke *et al.* 2009) and known to associate with induction of apoptosis (Espinosa *et al.* 2006) as well as impaired phagocytosis (Reefman *et al.* 2007).

11 Pathogenesis

The pathogenesis of CLE is not completely understood, but it is thought that development of the skin disease is related to the same autoimmune abnormalities responsible for the systemic disease (Rothfield *et al.* 2006). Although there is a link between skin and systemic manifestations, often skin may flare up independently or patients may have SLE without skin disease (Werth 2007). Treatments also may improve the skin without an effect on systemic disease suggesting that there are pathogenic differences. The immune dysfunction seems to be more important for SLE (Mok and Lau 2003), while exogenous agent(s) leading to skin inflammation may be central in CLE (Lee and Sinha 2006; Lin *et al.* 2007).

11.1 Pathogenesis of cutaneous lupus erythematosus

As basal keratinocytes are the primary focus of injury seen in CLE, skin may be the site of autoimmune initiation. It has become evident that UV radiation exposure significantly compromises the immune system and immunosuppressive effects are manifold (Kuhn *et al.* 2005). A potential pathogenic model where UV is the key player was first proposed by Norris (1993) (Figure 1). In this model exposure to UV light induces apoptosis of keratinocytes and the release of proinflammatory cytokines (reviewed in Kuhn and Bijl 2008). The presence or even accumulation of apoptotic cells results in the induction of inflammatory skin lesions. Emerging new evidence adds enhanced type 1 interferon (IFN) production into the CLE pathogenesis (Wenzel and Tuting 2007; Wenzel and Tuting 2008; Wenzel *et al.* 2009). Based on this model, several factors are required concurrently for CLE to develop, namely abnormal reactivity to UV light, the presence of antibodies and the presence of activated lymphocytes (Lin *et al.* 2007). The autoimmune response in skin can be viewed in three phases: initiation, immune response amplification and target damage.

In the *initiation phase*, UV exposure is responsible for enhanced keratinocyte apoptosis and translocation of autoantigens, such as Ro/SSA and DNA, to the cell surface, resulting in increased autoantigen display to the immune system. Genetically determined malfunctions in apoptosis or clearance system may further accumulate antigenic load. This setting, coupled with a UV-induced proinflammatory cytokine release, is a proper environment for the break in immune tolerance directed at self-tissues. (Lee and Sinha 2006).

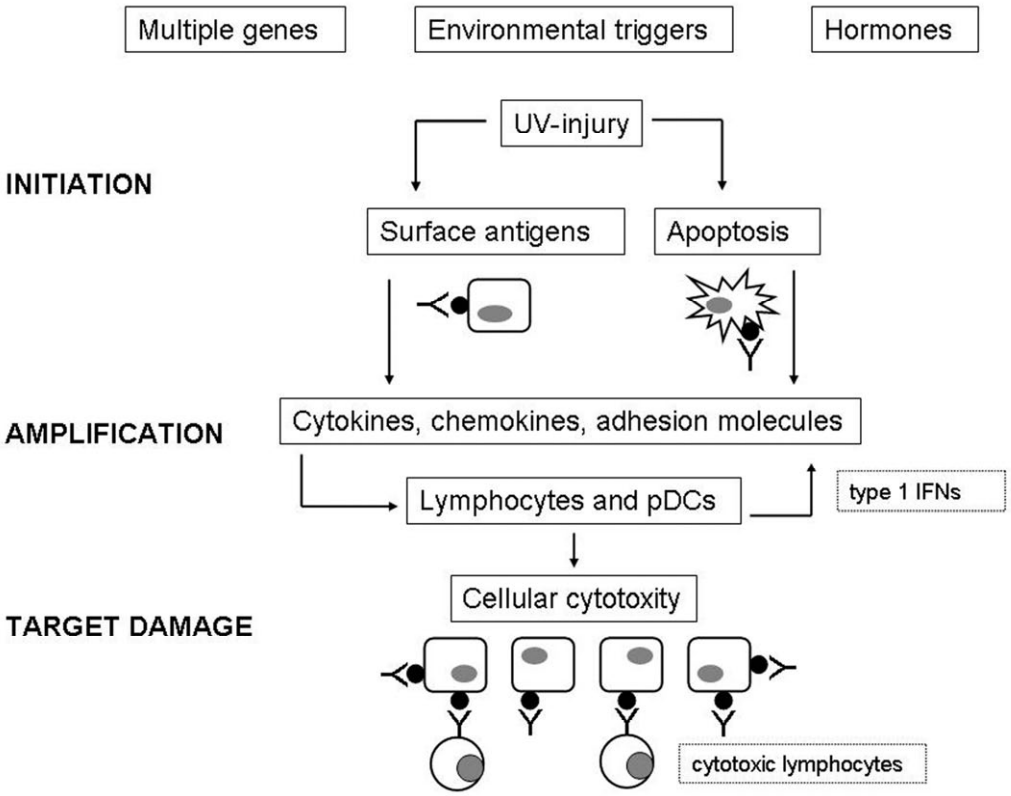

Figure 1. The three phases of CLE pathogenesis. UV light stimulates cell surface expression of Ro/SSA autoantigens and keratinocyte apoptosis. The release of adhesion molecules leads to migration of lymphocytes and plasmacytoid dendritic cells (pDC) that produce more effector cytokines amplifying chemokine production and cell recruitment. Circulating Ro antibodies bind to keratinocytes, cytotoxic lymphocytes infiltrate the dermis and destroy basal keratinocytes. IFN, interferon.

Amplification of the immune response is orchestrated by cytokines that induce local mediators of inflammation, including adhesion molecules, selectins and chemokines. Adhesion molecule expression may account for the increased homing of lymphocytes to the skin and the keratinocyte targets. (Lee and Sinha 2006). Mediator molecules also recruit and activate dendritic cells that release type 1 IFNs (Lin *et al.* 2007), which together with the cellular attack on keratinocytes, may promote further cytokine release to perpetuate and amplify the autoimmune response (Lee and Sinha 2006).

Deposition of immune complexes in tissues and subsequent autoantibody response are supposed to cause *target damage* (Lee and Sinha 2006). Another mechanistic explanation is antibody-dependent cellular cytotoxity, in which circulating Ro/SSA autoantibodies bind to keratinocytes expressing surface Ro autoantigen, thereby facilitating infiltration of cytotoxic lymphocytes and destruction of basal keratinocytes (Lee and Sinha 2006; Lin *et al.* 2007).

11.2 Pathogenesis of systemic lupus erythematosus

Production of harmful autoantibodies is the primary immunological disturbance and cause of tissue injury in SLE. A plausible disease model has been suggested (Mok and Lau 2003), and has been recently refined by placing the susceptibility genes in the pathogenetic context (Rhodes and Vyse 2008). Many factors are thought to be involved in the production of autoantibodies: defective immune regulation, such as errant clearance of apoptotic cells and immune complexes; dysregulation of T- and B-cells resulting in loss of self-tolerance; as well as cytokine milieu imbalance (Mok and Lau 2003). All these phenomena, in turn, are a consequence of multiple genes, precipitating environmental factors and gender effects. Several of the identified genes predisposing to SLE have functions related to these pathways (see section 14.2.1 and Figure 3).

11.3 Type 1 interferons

Type 1 IFNs are a family of cytokines produced by plasmacytoid dendritic cells (pDC) (Ronnblom and Pascual 2008). They bind to common receptor complexes and initiate a signalling cascade leading to the transcription of IFN-inducible genes (Kyogoku and Tsuchiya 2007). Patients with SLE have an increased espression of type 1 IFN regulated genes because of continuous production of IFN-α (Ronnblom and Pascual 2008). A similar upregulation of IFN-induced genes has also been demonstrated in the skin and blood of patients with DLE (Lee and Sinha 2006). IFN-α is important in the maturation of dendritic cells and the production of proinflammatory cytokines, and has diverse immunomodulatory effects, including stimulation of the T helper cell (Th) 1 pathway, promotion of B-cell activation and control of apoptosis (Ronnblom and Pascual 2008). Furthermore, elevated serum IFN-α levels in patients with SLE correlate with disease activity and severity. In SLE, a type 1 IFN driven disease model has been proposed in which unabated activation of pDCs sustains constitutive IFN-α production and further activation of autoreactive T- and B-cells (Kyogoku and Tsuchiya 2007; Ronnblom and Pascual 2008). Studies on genes in this pathway have identified *IRF5*, *TYK2* and *STAT4* as risk genes in SLE susceptibility (Remmers *et al.* 2007; Sigurdsson *et al.* 2005).

11.3.1 Type 1 interferons in cutaneous lupus erythematosus

It has become evident that the type 1 IFN system plays an important role in the development of CLE skin lesions (Wenzel and Tuting 2007; Wenzel and Tuting 2008; Wenzel *et al.* 2009). Patients with CLE are found to have large numbers of pDCs in their skin lesions (Wenzel and Tuting 2007). It was further shown that the expression pattern of IFN-induced MxA protein

reflected the characteristic distribution of the inflammatory infiltrate seen in CLE (Wenzel *et al.* 2007) and that expression was upregulated in patients with acute widespread lesions (Wenzel *et al.* 2005). It has been suggested that production of type 1 IFNs induces Th1-biased inflammation through induction of IFN-inducible chemokines leading to the recruitment of chemokine receptor expressing T-cells into skin lesions (Wenzel *et al.* 2005). That the IFN-induced proteins and chemokines are expressed on the same areas where cytotoxic lymphocytes invade the basal dermis and cause keratinocyte death and that peripheral lymphocytes expressing the chemokine receptor are decreased in numbers support the cellular immune response theory (Wenzel *et al.* 2005; Wenzel and Tuting 2008). Lesional induction of type 1 IFNs appears to be a hallmark of skin inflammation, but the primary stimulus is still unclear. It has been proposed that the accumulation of apoptotic cells in CLE skin and subsequent release of proinflammatory compounds may trigger skin lesions (Wenzel and Tuting 2008). The chronic inflammation may be further amplified by self-perpetuating mechanisms (Wenzel and Tuting 2007).

12 Genetic mapping in complex diseases

Completion of the human genome sequence in 2001 (Lander *et al.* 2001; Venter *et al.* 2001), launching the International HapMap Project (International HapMap Consortium 2003; International HapMap Consortium *et al.* 2007; http://www.hapmap.org) in 2002, an attempt to catalogue all variation in the human genome by the 1000 Genomes Project (http://www.1000genomes.org), development of elegant high-throughput genotyping techniques and massive publicly available databases of genetic information have all opened new doors to map genetic variants underlying complex diseases, such as LE. Genetic mapping of complex diseases, denoted as localisation of genetic factors underlying phenotypes on the basis of correlation with DNA variation, has turned out to be a challenging task based on a number of reasons. Complex diseases are not inherited in a straightforward Mendelian, monogenic fashion (Criswell 2008). This implies that there are multiple common genetic factors (f>5%) for the disease, each of which has modest increments in risk (i.e. odds ratios (OR) are usually 1.1-1.5), and explains only a small fraction of heritability (Altshuler *et al.* 2008). The missing heritability or “dark inheritance” not identified by current genome-wide strategies is thought be composed of low-frequency (0.5-5%) variants that increase disease risk two to three-fold and could contribute substantially to unexplained heritability, and rare variants (f<0.5%) of small effect (Manolio *et al.* 2009). Inherited genotype does not always correspond to resultant phenotype due to incomplete penetrance of predisposing loci or disease may develop in the absence of apparent genetic risk factors. Not all affected individuals for a particular disease share the same genetic risk (genetic heterogeneity), and finally, there remains an important role for interacting environmental factors (see section 14.4) (Criswell 2008).

Complex diseases may be relatively common in a given population. In general, they often have a late onset, hence having little or no impact on reproductive fitness, which allows causative alleles to rise to moderate frequency in the population. It is also believed that some alleles, being advantageous or neutral during human evolution, might now confer

susceptibility to diseases because of changes in living conditions accompanying civilisation. Disease-causing alleles could be maintained at high frequency when the disease burden is counterbalanced by a beneficial phenotype. In accordance with this, the "common disease-common variant" hypothesis was postulated, which suggests that common polymorphisms having a minor allele frequency (MAF) over 1% might contribute to susceptibility to common diseases (reviewed in Altshuler *et al.* 2008). This does not necessarily mean that all causal mutations are common, only that some common variants exist and could be used to pinpoint loci for detailed study.

Before 2006 the standard principle to identify susceptibility genes was either through positional mapping approaches or by functional candidate gene studies. Positional mapping approaches are hypothesis-free and consist of two steps: initial linkage studies in families with multiple affected individuals provide information on both the position and genetic effect of underlying disease loci, while association mapping extends linkage analysis to map the position of a disease gene to a much higher resolution (Todd 2001; Wang *et al.* 2003). The candidate gene method aims to identify candidate genes based on prior knowledge of the disease pathogenesis, functions of the selected genes, and in some cases, data on animal models of the disease (Wang *et al.* 2003). Utilising these two methods, *MAMDC1* and *GIMAP5* genes, respectively, were identified as disease-causing genes in Finnish SLE families (Hellquist *et al.* 2007; Hellquist *et al.* 2009).

In general, genetic linkage and association analysis rely on similar principles, that is, coinheritance of adjacent DNA variants. Linkage identifies alleles or haplotypes (i.e. stretches of alleles) that are inherited intact over several generations in families; association relies on the retention of adjacent DNA variants close to disease causing mutation over many generations (Cardon and Bell 2001). Thus, association studies can be regarded as very large linkage studies of unobserved, hypothetical pedigrees. Following the introduction of high-throughput genotyping technology it is possible today to genotype a million single nucleotide polymorphisms (SNP) in one individual at a time, which has led genetic research into the genome-wide association study (GWAS) era. The GWA analysis combines the power and resolution of a traditional association study with the hypothesis-free methodology of a conventional genome-wide linkage scan. In 2006, eight GWASs were published, the number of which had grown to 536 by September, 2009 (Hindorff *et al.* 2010; http://www.genome.gov/gwastudies), and GWAS is now the most widely used approach for genetic mapping (McCarthy *et al.* 2008).

12.1 Association analysis

Association analysis is more powerful than a linkage study to detect genetic contributions to complex diseases (Risch and Merikangas 1996) and is based on the comparison of differences in allele frequencies between cases and appropriate controls. The control individuals can either be unrelated, matched individuals or unaffected family members. Contrary to linkage analysis that focuses on recent recombination events, association looks for historical recombination within populations across hundreds of thousands of generations (Cardon and

Bell 2001). The most common strategy for gene identification by association is the case-control study design (McCarthy *et al.* 2008). The advantage of a case-control study is that sporadic patients are obtained more easily than families and that this method is a powerful strategy for identifying genes of small effects that contribute to complex traits (Cardon and Bell 2001; Risch 2000). Despite its ease, association strategy has encountered many limitations, including lack of reproducibility of results, insufficient sample size, poor study design, poorly matched controls and unaccurate recording of phenotypic information (Cardon and Bell 2001). The problem of spurious associations is explained by population stratification, meaning that individuals with different ancestral and demographic histories, and therefore different allele frequencies independent of the disease, are present in the population (Cardon and Bell 2001; McCarthy *et al.* 2008). To overcome the problem of population stratification, various approaches have been developed that use controls selected from the families of affected individuals. Basically, these methods use the parental alleles or haplotypes that are not transmitted to affected offspring as the control sample (Borecki and Province 2008). The most popular approach to account for stratification is the transmission disequilibrium test (Spielman *et al.* 1993).

12.2 The genome-wide association study approach

The GWAS approach is based on association but does not rely on any prior hypothesis regarding position or function and aims to capture a substantial proportion of common variation in the human genome by using knowledge of linkage disequilibrium (LD) relationships. The publication of GWAS of 14,000 cases and 3,000 controls by Wellcome Trust Case Control Consortium (2007) can be considered as a fundamental step into the GWA era and this approach is now routinely used in genetic research of complex diseases. Even though GWASs have been very successful in identifying susceptibility genes, there are still limitations. First, GWASs generally identify only common genetic variants and the studies performed thus far have had good power to detect alleles that are common in the general population and have modest effect sizes (Altshuler *et al.* 2008). Thus rare variants or those with small effect sizes are likely to have been missed in current GWAS designs. Second, the coverage of GWASs is nonrandom, meaning that subsets of genomic regions are poorly covered (Frazer *et al.* 2009). Third, in most cases the association signals identified in GWASs are likely to be indirect associations due to LD and not the causal variants themselves (Altshuler *et al.* 2008). Finally, GWASs have been criticised for focusing on populations of European ancestry and using other ancestries only for limited replication thus making the risk assessment in other populations problematic (Frazer *et al.* 2009). This addresses the importance of recruiting populations of different ethnicity for primary genotyping and the need for replication studies to unambiguously identify functional variant(s).

13 Genetic variation in the human genome

Genetic variations in the human genome can be either common or rare but obviously with a continuous distribution. Common variants are defined as genetic variants with a MAF of at least one percent in the population and are considered synonymous with polymorphisms. Rare variants occurring at less than one percent of frequency are generally defined as mutations. (Frazer *et al.* 2009). Genetic variation includes SNPs and simple sequence repeats, such as micro- and minisatellites, as well as structural variants, namely insertion-deletions (indels), block substitutions, inversions, copy number variants, segmental duplications and translocations (Feuk *et al.* 2006; Frazer *et al.* 2009) (Figure 2). The vast majority of genetic variants are hypothesised to be neutral, in other words, they do not contribute to phenotypic variation. However, the relative percentage of neutral and nonneutral variants awaits to be determined (Frazer *et al.* 2009). The spontaneous mutation rate of SNPs is estimated to be as low as $2.5x10^{-8}$ mutations per base and generation (Nachman and Crowell 2000) whereas genomic rearrangements, such as copy number variants, appear to be two to four orders of magnitude more prevalent (Lupski 2007).

13.1 Single nucleotide polymorphisms

The most abundant small-scale variation in the human genome is a SNP. They are base substitutions in which a single nucleotide is altered into another at a fixed position of the sequence (Figure 2). The number of SNPs within the human genome is approximately 11 million, meaning that there is one base substitution for every 300 nucleotides (Feuk *et al.* 2006; Frazer *et al.* 2009). It has been estimated that approximately 2% of the SNPs are biologically important (Orr and Chanock 2008). Nonsynonymous SNPs are located in the protein coding region of the gene and cause amino acid substitutions, frame shifts or termination of protein translation. Synonymous SNPs within exons do not alter protein primary structure, but can affect messenger RNA (mRNA) stability or alter splicing signals. SNPs outside the protein coding regions can also be of functional importance, either by their location on promoter regions where they may interfere with gene regulation by altering transcription factor binding sites, or by their location in enhancers or silencers.

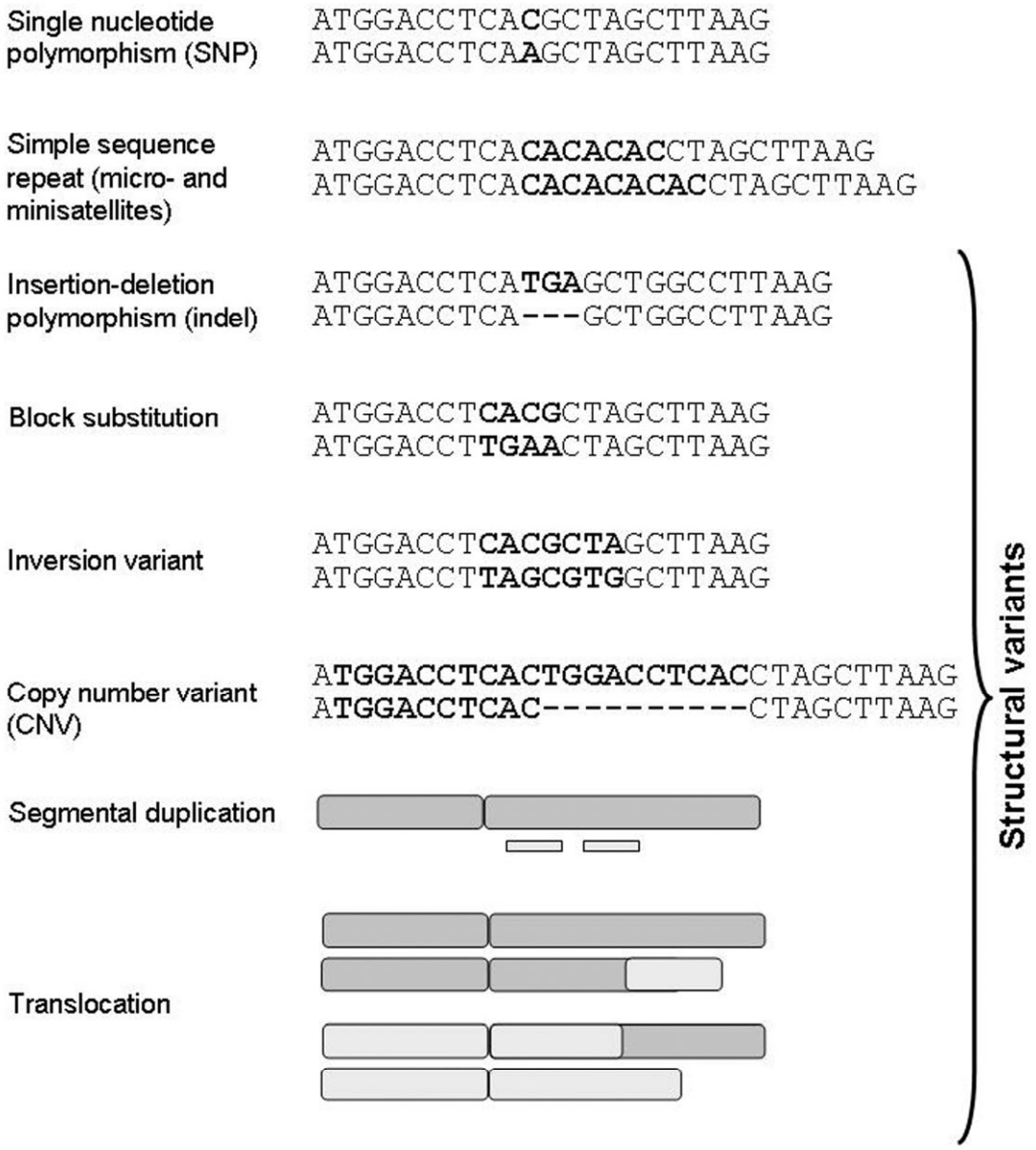

Figure 2. Classes of human genetic variation. DNA sequence variations affecting a single nucleotide are known as single nucleotide polymorphisms. Insertion-deletion variants occur when base pairs are present in some genomes but absent in others. Simple sequence repeats are short tandem repeat units, block substitutions are a string of adjacent nucleotides that varies between two genomes, while in an inversion variant the order of the base pairs is reversed in a defined section of a chromosome. Copy number variants occur when identical or nearly identical sequences are repeated in some chromosomes, while segmental duplications are repeated segments with near-identical sequence. Translocations are rearrangements of chromosomal sections between nonhomologous chromosomes. Modified from Frazer *et al.* (2009).

14 Genetics of lupus erythematosus

Burch and Rowell (1968) have hypothesised that the polygenic predisposition for the development of DLE is different from that of SLE: "*When a genuine transition from DLE to SLE occurs, the affected patient is genetically predisposed to both diseases*" (reviewed in Lee and Sinha 2006). The question of whether the genetic susceptibility to cutaneous or systemic disease is overlapping or distinct remains yet unsolved. Based on the evidence available to date, it is plausible that differential expression of multiple genes together with an array of environmental exposures leads to differential clinical expression of disease phenotypes. While the genetics of SLE has been extensively studied over the decades, the genetic architecture of CLE remains to be elucidated.

A strong genetic component underlies SLE and a similar genetic basis of inheritance likely holds true for CLE as well. SLE exhibits familial clustering with a sibling risk ratio (λ_s) as high as 29 (Alarcon-Segovia *et al.* 2005); in other words siblings of an affected individual are 29 times more likely to develop SLE than those without an affected sibling. Familial clustering has also been documented for DLE; the prevalence of DLE was 3.5% in 255 first degree relatives of patients compared to that of 0.5% in control families (Lawrence *et al.* 1987). Higher concordance rates between monozygotic twins with SLE (>35%) relative to dizygotic twins and other full siblings (2-5%) (Deapen *et al.* 1992) also support important roles for genes. The prevalence of CLE is too low for a formal concordance study with twin pairs (Millard and McGregor 2001). However, occurrence of DLE in monozygotic twins has been described in a few case reports (Steagall *et al.* 1962; Wojnarowska 1983 and refrences therein). The genetic component is marked by heritability estimates of 66% and 44% for SLE and DLE, respectively (Lawrence *et al.* 1987).

14.1 Susceptibility genes for cutaneous lupus erythematosus

A genetic predisposition is probably the greatest risk factor for CLE despite the multitude of environmental exposures. There are only a few confirmed risk loci in CLE, including human leukocyte antigen (*HLA*) genes, the *TNF-α* promoter and complement factors (Lee and Sinha 2006; Millard and McGregor 2001; Osmola *et al.* 2004). Many genetic regions outside the *HLA* locus appear to be involved in susceptibility to CLE or demonstrate association or linkage with the Ro/SSA autoantibody response. These include loci encoding cytokines and their receptors, adhesion molecules, antioxidant enzymes and apoptosis genes (Millard and McGregor 2001; Osmola *et al.* 2004) (Table 4). In addition, the non-*HLA* complement gene *C1QA* and DNA exonuclease *TREX1* have been implicated in CLE susceptibility (Racila *et al.* 2003; Rice *et al.* 2007). Many of these (*HLA* genes, complement components, IL-10, Fcγ receptor II, *C1QA* and *TREX1*) have been linked to SLE as well, supporting the theory of overlapping pathogenesis (Lee and Sinha 2006; Lee-Kirsch *et al.* 2007; Martens *et al.* 2009). Unfortunately, studies on CLE have been difficult to evaluate and replicate due to insufficient sample sizes and because risk estimates are seldom reported. Some of the CLE susceptibility genes are discussed in more detail below.

14.1.1 Insights into CLE susceptibility genes

The extremely diverse *HLA* region on chromosome 6p21.3 plays a vital role in the immune system. Historically, the *HLA* region has been subdivided into three classes that encode not only the HLA but also complement and TNF molecules. Several studies in SCLE have identified a common *HLA* susceptibility haplotype (*HLA*-A1, B8, DR3, DQ2) particularly in the presence of Ro/SSA autoantibodies (Sontheimer 2005). Relatively few studies conducted in DLE implicate an association with *HLA* (*HLA*-A1, B8, DR3 and B7, DR2 haplotypes), while others have not found such a relationship (Lee and Sinha 2006; Millard and McGregor 2001; Osmola *et al.* 2004). The mechanism by which *HLA* genes influence disease are not fully understood, but are likely to be related to their role in T-cell repertoire selection, antigen presentation and immune activation (Lee and Sinha 2006).

Table 4. Genes outside the *HLA* region suggested as being involved with CLE pathogenesis (modified after Millard and McGregor 2001; Osmola *et al.* 2004).

Group	Gene	Locus	Evidence[1]
Cytokines	IL-1, IL-10	2q13, 1q31	Photosensitivity, Ro/SSA production
Cytokine receptors	FCGR2, TCR Cβ1, Cβ2	1q23, 7q35	Ro/SSA production
Adhesion molecules	ICAM-1, E-selectin	19p13.3-p13.2, 1q23-25	Increased expression
Antioxidant enzymes	GSTM1	1p13	Ro/SSA production
Apoptosis genes	Fas, TREX1	10q24.1, 3p21.31	Photosensitivity, DNA damage
Non-HLA complement	C1QA	1p36.3-p34.1	SCLE phenotype

C1QA, complement component 1, q subcomponent, A chain; *FCGR2*, Fc fragment of IgG, low affinity IIa, receptor (CD32); *GSTM1*, glutathione S-transferase mu 1; *ICAM-1*, intercellular adhesion molecule 1; *IL*, interleukin; *TCR*, T-cell receptor; *TREX1*, three prime repair exonuclease 1

[1] Millard and McGregor (2001) and references therein; Racila *et al.* (2003); Rice *et al.* (2007)

Inherited deficiencies of C2 and C4 molecules have been strongly linked to DLE, SCLE and Ro/SSA response (Lee and Sinha 2006; Millard and McGregor 2001; Osmola *et al.* 2004). Proposed basis for these associations include a failure to clear immune complexes and apoptotic cells, but also a false positive relationship due to strong linkage disquilibrium (LD) (Millard and McGregor 2001; Osmola *et al.* 2004). SCLE patients homozygous for the *C1QA* gene variant (C1qA-Gly70$_{GGA}$) have lower serum levels of C1q protein compared to patients not carrying this variant (Racila *et al.* 2003). It is not known how the genetic variation results in functional abnormality and is related to pathogenesis, but defects in apoptotic clearance or solubilisation of circulating immune complexes are possible mechanisms (Racila *et al.* 2003).

Keratinocytes expressing the rare (f=15% in healthy population) *TNF-α* promoter polymorphism (-308A) exhibit greatly enhanced TNF-α production in response to UV light (Millard and McGregor 2001) and a strong association to photosensitive SCLE has been demonstrated (Werth *et al.* 2000). Interestingly, risk of developing DLE and prevalence of discoid lesions in SLE have been reported to be higher in subjects that produce low levels of TNF-α (Suarez *et al.* 2005). The pathogenic mechanism probably acts through translocation of antigens to cell surface and their exposure to the immune system (Werth *et al.* 2000). However, the independent role of *TNF-α* has been disputed due to the strong LD across the *HLA* region that makes identification of causal alleles difficult (Fernando *et al.* 2007; Millard *et al.* 2001).

Several studies have shown involvement of intercellular adhesion molecule 1 (*ICAM-1*) in the pathogenesis of various skin diseases, including different forms of CLE (Kuhn *et al.* 2002). Increased keratinocyte expression of ICAM-1 has been demonstrated in evolving UV-induced lesions in CLE, but not in healthy controls (Kuhn *et al.* 2002; Nyberg *et al.* 1999). Strong keratinocyte expression of ICAM-1 was already found one week after photoprovocation suggesting that these early changes reflect an underlying defect in the mechanisms that regulate adhesion molecule expression in LE (Nyberg *et al.* 1999). Sera obtained from SCLE patients display elevated levels of soluble ICAM-1 (Nyberg *et al.* 1997). Thus ICAM-1 itself or its upstream regulators are putative disease candidate genes. A recently identified new SLE susceptibility gene *ITGAM* is a receptor for ICAM-1 (Harley *et al.* 2008; Hom *et al.* 2008; Nath *et al.* 2008). Despite the supporting evidence, a candidate gene analysis of three photosensitive disorders failed to demonstrate association to *ICAM-1* (Millard and McGregor 2001), possibly due to a small number of samples leading to insufficient power.

The family of glutathione-S-transferase (GST) enzymes is involved in detoxification of hazardous foreign compounds (Hayes and Strange 2000). A number of genetic polymorphisms altering enzyme activity have been described at the four major *GST* loci, namely *GSTA*, *GSTM*, *GSTP* and *GSTT*. The *GSTP1* variant (I105V) has a protective effect in CLE patients with polymorphous light eruption (Millard *et al.* 2008), while homozygosity of *GSTM1* null alleles is associated with Ro/SSA autoantibody production in SLE patients (Ollier *et al.* 1996). Interestingly, *GSTs* have shown to interact with environmental exposures in SLE (Fraser *et al.* 2003; Karlson *et al.* 2007). Moreover, *GST* polymorphisms seem to account for the level of chromosomal damage, formation of DNA adducts and tobacco consumption in individuals who smoke (Jourenkova *et al.* 1998; Piipari *et al.* 2003; Tuimala *et al.* 2004).

14.2 Susceptibility genes for systemic lupus erythematosus

The genetics of SLE has been studied since the early 1970s when the first *HLA* allele association was found (Harley *et al.* 2006). The following four decades have been witness to a tremendous genetic technological revolution facilitating the identification of susceptibility loci and genes for complex diseases, including SLE. When this doctoral study was started in 2006, there were nine linkage regions found in SLE that have reached the threshold (LOD-

score >3.2) for significant linkage (Harley *et al.* 2006). Four years later in 2010, there are more than 30 known disease contributing genes of which over half have been discovered in the past three years underscoring the success of new genetic mapping approaches (Moser *et al.* 2009).

The linkage regions established and independently confirmed by genome-wide scans include 1q23, 1q31-32, 1q41-41, 2q37, 4p16, 6p21-p12, 11p13, 12q24, 16q12-13 (reviewed in Harley *et al.* 2006; Rhodes and Vyse 2007). Linkage on chromosomes 5p15-5q11 and 14q21-q23 had been implicated (Gaffney *et al.* 1998; Koskenmies *et al.* 2004; Lindqvist *et al.* 2000; Namjou *et al.* 2002a; Shai *et al.* 1999), but the disease contributing gene *MAMDC1* on chromosome 14 had not been identified until recently (Hellquist *et al.* 2009). Two meta-analyses have further identified two significantly linked regions, namely 20p11-q13.13 (Forabosco *et al.* 2006) and 16p12-16q13 (Lee and Nath 2005). A subsequent fine-mapping of the 16p12-16q13 region recently identified association to the integrin alpha M (*ITGAM*) gene, also known as *CD11b* or complement receptor 3, which is expressed primarily on neutrophils, macrophages and dendritic cells and can bind a variety of ligands, including ICAM-1 (Hom *et al.* 2008; Nath *et al.* 2008). Simultaneously, this gene was shown to be strongly associated to SLE in two of the GWAS (Harley *et al.* 2008; Hom *et al.* 2008) and its robust role has been confirmed thereafter in several studies encompassing different populations (Han *et al.* 2009b; Molineros *et al.* 2009; Yang *et al.* 2009).

Before the introduction of GWASs there were nine established susceptibility genes identified through candidate gene studies or linkage approach (Moser *et al.* 2009), including *HLA* locus, complement locus, *TNF-α*, *FcγRIIA* and *FcγRIIIA*, *PDCD1*, *PTPN22*, *IRF5*, *STAT4* and *TREX1*. Other genes, such as *TYK2* and *CTLA4*, requiring further work have either low levels of significance attached to them or the existing results are inconsistent (Rhodes and Vyse 2008). Applying the hypothesis-free whole genome strategies to the hunt for SLE genes has considerably broadened our understanding of the genetic basis and biochemical pathways of this disease. At the beginning of 2008, three high-density GWASs (Graham *et al.* 2008; Harley *et al.* 2008; Hom *et al.* 2008) and one smaller study (Kozyrev *et al.* 2008) were published. Together, the studies have evaluated more than 5,000 patients, a number that had only been a wild dream a couple of years before. One additional GWAS in Han Chinese (Han *et al.* 2009a), two replication studies (Gateva *et al.* 2009; Suarez-Gestal *et al.* 2009) and one meta-analysis (Graham *et al.* 2009) have caused the number of convincing genetic associations to swell to well over 30. The vast majority of the susceptibility genes fall into key biological pathways shown to be important in the disease pathogenesis, including immune complex clearance, immune signal transduction and interferon pathways, but there is also a group of genes without any apparent immunological role or assigned function (Figure 3). Not all of the newly described findings, however, have attained high levels of confirmation and further work is required to clearly establish their effects and key polymorphisms (Rhodes and Vyse 2008). Presenting all identified genes is beyond the scope of this literature review. However, some of the genes placed in the context of disease pathways are discussed below, while a more comprehensive picture is given in recent review articles (Harley *et al.* 2009; Moser *et al.* 2009; Rhodes and Vyse 2008).

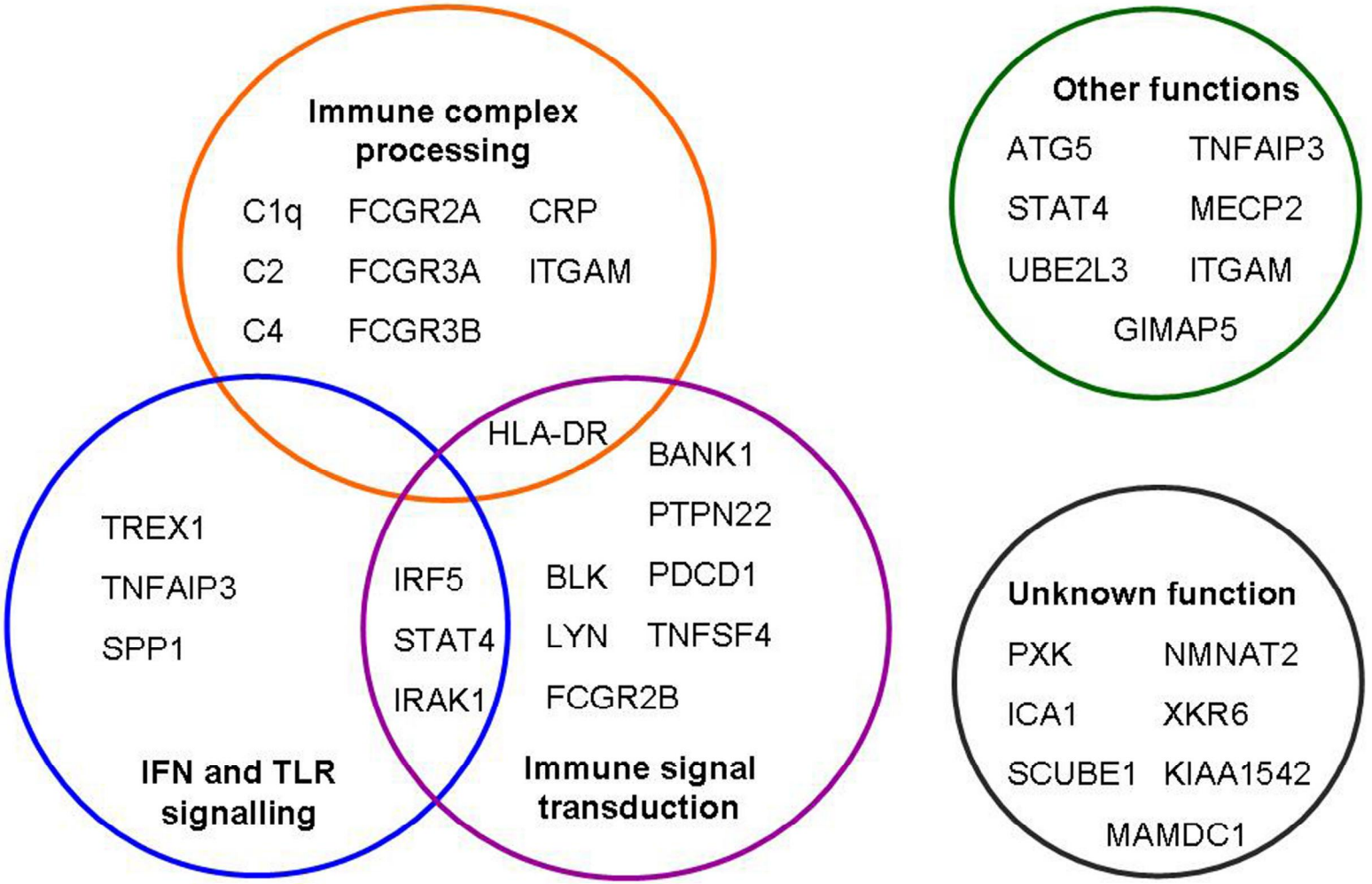

Figure 3. Immune function pathways that contain established candidate genes involved in systemic lupus erythematosus. Genes involved in each pathway are indicated. TLR, toll-like receptor. Modified from Moser *et al.* (2009); Harley *et al.* (2009).

14.2.1 Insights into SLE susceptibility genes

Interferon regulatory factor 5 (*IRF5*) and tyrosine kinase 2 (*TYK2*) are both members of the IFN-Toll like receptor signalling pathway together with *STAT4* and *TNFAIP3*. After the identification of *IRF5* and *TYK2* in SLE susceptibility (Sigurdsson *et al.* 2005), *IRF5* has sparked a flurry of confirmation and characterisation reports not only in SLE but also in other diseases, namely rheumatoid arthritis (RA), inflammatory bowel disease, SS and multiple sclerosis (Dideberg *et al.* 2007; Kristjansdottir *et al.* 2008; Miceli-Richard *et al.* 2007; Sigurdsson *et al.* 2007). In contrast, *TYK2* has remained a somewhat controversial finding (Graham *et al.* 2007; Harley *et al.* 2008). The *IRF5* gene encodes a transcription factor involved in the control of inflammatory and immune responses. Thus, genetic variants altering the actions of *IRF5* could result in a prolonged proinflammatory response in genetically susceptible individuals (Rhodes and Vyse 2008). Four functional polymorphisms have been described: rs200460 creating a donor splice site (Graham *et al.* 2006); rs10954213 altering polyadenylation signal and transcript stability (Cunninghame Graham *et al.* 2007); 30 bp indel likely to influence transcription initiation of target genes (Graham *et al.* 2007; Moser *et al.* 2009); and finally 5 bp CGGGG indel upstream of the first untranslated exon creating an additional SP1 binding site (Sigurdsson *et al.* 2008a). It has been claimed that this latter polymorphism explains the association signal arising from multiple SNPs in the *IRF5* gene and a general role in autoimmune disorders has been suggested (Sigurdsson *et al.* 2008a). TYK2 is an intracellular signalling molecule that interacts with the IFN-α receptor (Rhodes and Vyse 2007). No functional data exist for the detected polymorphisms in *TYK2*, but their location implicates altered protein function or missplicing events (Graham *et al.* 2007; Sigurdsson *et al.* 2005). Deficiency of TYK2 leads to defects of multiple cytokine pathways, including type 1 IFN, IL-6, -10, -12 and -23 and to an accelerated humoral immune response (Minegishi *et al.* 2006).

Signal transducer and activator of transcription 4 (*STAT4*) was simultaneously demonstrated in RA and SLE (Remmers *et al.* 2007) and has been confirmed thereafter by independent replication studies (Abelson *et al.* 2009; Sigurdsson *et al.* 2008b; Taylor *et al.* 2008), also in the Finnish SLE family cohort (Hellquist *et al.* 2009) and in SS (Korman *et al.* 2008). Furthermore, common polymorphisms of *STAT4* predispose to more severe disease manifestations (Hellquist *et al.* 2009; Sigurdsson *et al.* 2008b; Taylor *et al.* 2008). Studies also demonstrate that *STAT4* acts by an additive manner with *IRF5* (Abelson *et al.* 2009; Nordmark *et al.* 2009; Sigurdsson *et al.* 2008b). *STAT4* encodes a transcription factor that is induced by cytokines, including type 1 IFNs, IL-6 and IL-23. After activation it promotes differentiation of IFN-γ producing Th1 cells, and possibly that of Th17 cells (Rhodes and Vyse 2008), which play a role in a wide range of autoimmune diseases (Pernis 2009).

Tumour necrosis factor-α-induced protein 3 (*TNFAIP3*) was discovered and replicated in two sequential studies (Graham *et al.* 2008; Musone *et al.* 2008). *TNFAIP3* encodes an enzyme required for the downstream signal transduction of Toll-like receptors and is a negative regulator of nuclear factor-κB pathway, thus playing a key role in modulating a broad range of cellular functions, including cell activation, cytokine signalling and apoptosis (Graham *et*

al. 2008). Preliminary functional experiments demonstrate that SLE risk is associated with reduced inhibition of nuclear factor-κB activity (Musone *et al.* 2008).

Identification of three genes, namely *BANK1*, *BLK* and *LYN* (Harley *et al.* 2008; Hom *et al.* 2008; Kozyrev *et al.* 2008), involved in B-cell signalling attested the importance of this pathway in SLE pathobiology. B-cell scaffold protein with ankyrin repeats 1 (*BANK1*) is a B-cell adapter protein expressed predominantly on B-cells and is thought to sustain B-cell activity, thus increasing disease risk (Kozyrev *et al.* 2008). LYN is a protein tyrosine kinase that physically associates the B-cell receptor with is binding partner BANK1, while BLK affects functions associated with the development of B-cells before the appearance of the B-cell receptor (BCR) (Moser *et al.* 2009). All these genes play a critical role in controlling the activation of B-cells following signalling through BCR (Rhodes and Vyse 2008). After ligand binding and BCR aggregation, an early intracellular event is the recruitment and activation of SCR family protein tyrosine kinases, including BLK and LYN, which mediate further intracellular signalling. BANK1 acts downstream of LYN, thus facilitating release of intracellular calcium, which is an important event in B-cell activation. Cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) belongs functionally to the same category of lymphocyte signalling with *BANK1*, *BLK* and *LYN*. It is a member of the immunoglobulin family of receptors that downregulates activated T-cells (Rhodes and Vyse 2008). *CTLA4* association to SLE has mainly relied on meta-analysis (Barreto *et al.* 2004; Lee *et al.* 2005) and its independent role has been less confident until recently (Graham *et al.* 2006). Interestingly, the association pattern appears to be disease-specific in different autoimmune diseases (Graham *et al.* 2006; Zhernakova *et al.* 2005).

14.3 Shared genetic predisposing factors in autoimmune diseases

Extensive concomitance of autoimmune disease has been reported in some patients and families (Zhernakova *et al.* 2009). This clustering may be explained by a common set of general susceptibility genes shared among family members, while disease-specific genes and environmental factors may dictate the final disease phenotype (Rhodes and Vyse 2007; Wong and Tsao 2006). The *HLA* region is a classical example of shared genetic basis of autoimmunity. Linkage scans already revealed considerable overlap in chromosomal location of linkage regions between multiple autoimmune diseases (Wandstrat and Wakeland 2001) and common genes were pinpointed, including *CTLA4*, *PDCD1*, *PTPN22* and *NOD2/CARD15* in type 1 diabetes, RA and SLE (Rioux and Abbas 2005). GWASs have thereafter brought to light several candidate genes many of which are shared between different autoimmune diseases and tend to cluster around the same immunological pathways (Lettre and Rioux 2008; Zhernakova *et al.* 2009). Examples include *STAT4* in RA and SLE; and *TNFAIP3* in RA, celiac disease and SLE. Clustering of the genes suggest shared underlying causal mechanism and further our understanding of the development and concomitance of these diseases. The lack of genetic overlap, on the other hand, suggests distinct disease mechanisms (Rhodes and Vyse 2008).

14.4 Gene-gene and gene-environment interactions

Gene-gene interaction, or epistasis, is a phenomenon that occurs when the effect of one locus is altered by the effect of another locus, that is, the effect of carrying more than one variant is different than would be expected by simply combining the effects of each individual variant (Cordell 2002; Hirschhorn and Daly 2005). Genetic interaction of distinct genes may result in multiple disease pathways with a variety of disease phenotypes that vary in severity (Wong and Tsao 2006). Consequently, diseases are of different intensities between individuals and in different ethnicities. From the mathematical point of view, epistasis is defined as deviation from additivity in a linear model (Moore and Williams 2009). Hence, statistical interaction does not necessarily imply interaction on the biological level and thus may not easily translate into physical interaction between proteins (Cordell 2002; Moore and Williams 2005). Furthermore, standard employment of linear models in genetic epidemiology challenges the identification of gene-gene interactions because these models have limited ability to detect nonlinear patterns of interaction (Moore and Williams 2009).

Disease development and course may be further influenced by interacting genes and environmental exposures. One example of such interactions in LE is UV light leading to an increased load of apoptotic material in an individual with genetically less efficient clearance mechanisms (Jonsen *et al.* 2007). *GST* polymorphisms have shown to interact with environmental exposures, resulting in increased risk and earlier diagnosis of SLE (Fraser *et al.* 2003; Karlson *et al.* 2007). N-acetyl transferase gene, in combination with chemical exposure, may also increase susceptibility to SLE (Jonsen *et al.* 2007). Identification of these interactions might be beneficial in disease prevention and treatment targeting. In order to effectively study gene-environment interactions several problems are to be overcome: time frame of environmental influence is protracted; exposure(s) can already take place *in utero* and may also interact with each other temporally; and large study cohorts with representative controls are needed (Jonsen *et al.* 2007). Retrospective studies are prone to recall bias whereas prospective studies are more accurate, but time-consuming (Costenbader and Karlson 2006). Finally, epigenetic mechanisms (i.e. chromatin changes that alter gene expression without affecting DNA sequence) possibly being targets of environmental factors may mediate the interaction between genes and environment (Jonsen *et al.* 2007).

15 Matrix metalloproteinases

Matrix metalloproteinases (MMP) constitute a large family of 24 zinc-dependent multifunctional, proteolytic enzymes that degrade extracellular matrix and basement membrane components (Overall and Lopez-Otin 2002). They also play a role in several pivotal cellular processes, including cellular growth, apoptosis, tissue repair, angiogenesis, inflammation and immunity (Parks *et al.* 2004). MMPs are divided into six subgroups according to their structure and substrate preference, namely collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-13, -10), matrilysins (MMP-7, -26), membrane-type MMPs (MMP-14, -15, -16, -17, -24, -25) and other MMPs (MMP-19, -20, -21, -27, -28) (Overall and Lopez-Otin 2002). Proteolytic activity of MMPs is regulated at several levels:

gene expression, pericellular localisation, as well as enzyme activation and inactivation (Parks *et al.* 2004). Typically these proteins are not expressed in normal healthy tissues, by contrast, expression is detected in diseased or inflamed tissues (Parks *et al.* 2004). Under normal physiological conditions, extracellular matrix remodelling occurs in a precisely controlled manner, whereas during the course of the disease in part in an attempt at repair, but is frequently dysregulated and destructive. MMP activity is blocked by general inhibitors, such as α2-macroglobulin, or specifically by tissue inhibitors of metalloproteinases (TIMP). There are four endogenous human TIMPs identified, including TIMP-1, -2, -3 and 4, which block MMP function by binding to the catalytic sites. Results indicate that TIMP-3 is a major regulator of metalloproteinases *in vivo*. In addition to MMP inhibition, TIMPs are involved in a broad range of other biological activities, such as apoptosis. Lack of efficacy and untoward side effects still constrain other exogenous approaches to inhibit metalloproteinase activity. (Murphy and Nagase 2008).

15.1 Matrix metalloproteinases in lupus erythematosus

Specific MMPs have been implicated in the aetiology of SLE, but their exact role in cutaneous disease is as yet unknown. One small-scale study has been conducted in which expression of MMP-3 was absent in the regions of basal layer destruction in DLE specimens, suggesting that MMP-3 may be involved in the pathogenesis of interface dermatitis (Gunduz *et al.* 2006). MMP-9 was diffused throughout the entire epidermis and was also detected in inflammatory infiltrate cells. Experimental evidence demonstrates a wide range of functions for both MMP-9 and MMP-3 in LE disease progress. MMP-9 protein activity is increased in the sera and plasma of SLE patients (Chang *et al.* 2008; Faber-Elmann *et al.* 2002). It may serve as a potential biomarker in neuropsychiatric SLE (Efthimiou and Blanco 2009), since high intrathecal and serum levels of MMP-9 have been detected in diseased individuals (Ainiala *et al.* 2004; Trysberg *et al.* 2004). Upregulation of this protein may also be responsible for the vascular damage seen in SLE peripheral neuropathy (Mawrin *et al.* 2003). It has also been documented that MMP-9 levels inversely correlate with dsDNA autoantibodies in affected individuals (Jiang *et al.* 2009; Makowski and Ramsby 2003). Interestingly, cultured peripheral blood mononuclear cells from SLE patients experiencing remission (amelioration) of their disease secrete more MMP-9 than cells obtained from relapsed (exacerbated) patients (Matache *et al.* 2003). In pregnant SLE women the disease may worsen in 60% of the cases. However, low levels of MMP-9 activity similar to those of control individuals were observed in patients (Munoz-Valle *et al.* 2003). Individuals affected with SLE have increased serum plasma levels of MMP-3 not correlating with disease activity (Zucker *et al.* 1999). Upregulation of the MMP-3 protein may contribute to vessel wall damage in SLE neuropathy (Mawrin *et al.* 2003), and increased serum levels of MMP-3, as well as MMP-9, may play a role in the pathogenesis of SLE nephritis (Jiang *et al.* 2009; Kotajima *et al.* 1998). Sera samples obtained from SLE patients display MMP-19, which may have a role in arthritis-associated tissue destruction (Sedlacek *et al.* 1998). Serum levels of TIMP-1 are reported to be comparable or even lower in SLE patients compared to healthy controls (Jiang *et al.* 2009; Matache *et al.* 2003; Robak *et al.* 2006). Decreased TIMP-1 expression in peripheral blood mononuclear cells suggests increased MMP activity in target

tissues (Mandel *et al.* 2004) and detected serum imbalance between MMP-9 and its main inhibitor TIMP-1 may contribute to the pathogenesis of lupus nephritis (Jiang *et al.* 2009). Taken together, these results suggest that MMPs, as well as TIMPs, may have important and multiple roles in the progression of inflammation and tissue damage in various rheumatic diseases including LE (Mandel *et al.* 2004).

15.2 Matrix metalloproteinases in apoptosis

Some MMPs function in apoptosis either directly or indirectly regulating survival signals generated by specific adhesive events. Actions can be both apoptotic (MMPs-1, -2, -3, -7, -9, -11) and antiapoptotic (MMPs-2, -3, -7, -9, -11). Proteolysis leads to enhancement or attenuation of apoptosis in a context dependent manner, in which the proteolytic cascade and cell fate is influenced by concentrations, tissue specificity and balance between MMPs and TIMPs. (Mannello *et al.* 2005). In terms of LE, MMP-7 mediates the release of the Fas ligand, a well known death factor, implicated in controlling mesangial inflammation in lupus nephritis (Tsukinoki *et al.* 2004). Endothelial cell apoptosis and survival of epithelial cells is influenced by MMP-14 (Langlois *et al.* 2005; Nagavarapu *et al.* 2002). Furthermore, TIMP-3 may promote apoptosis of keratinocytes (Salmela *et al.* 2003).

AIMS OF THE STUDY

The broad aim of this study was to identify genetic variants conferring susceptibility to CLE, and investigate whether genetic susceptibility is shared between clinically different conditions, that is, cutaneous and systemic variants of LE. The specific aims of the study were:

1. To epidemiologically characterise different subtypes of LE in order to better understand their clinical manifestations, typical laboratory aberrations, systemic symptoms, disease associations and triggering factors (I).

2. To test whether SLE candidate genes that were published prior to this study would constitute a risk in Finnish patients, and whether their combinations show evidence of epistasis, i.e. gene-gene interaction (II, III). In addition, the tissue expression of proteins encoded by the best associating genes was determined (II).

3. To evaluate the role of *ITGAM* in CLE (IV), and screen other GWAS-identified SLE susceptibility genes for their association to CLE (unpublished results).

4. To perform subphenotype analyses to study the correlation between relevant clinical variables and genotypes (II, III, IV).

5. To study the function and expression of certain MMPs and their specific inhibitors, TIMPs, in the skin lesions of CLE patients. Especially MMPs known to regulate apoptosis and induced by UV light were investigated (V).

MATERIALS AND METHODS

1 Study subjects and tissue samples

1.1 Ethical considerations

This study was based on large sample collections representing Finnish and Swedish patients with their respective control individuals. All participants gave written informed consent for participation in genetic studies and the study protocols were reviewed and approved by the Ethical Review Boards of Helsinki and Tampere University Central Hospitals, Finland, and the Regional Human Ethics Committee at the Karolinska University Hospital, the Institutional Ethics Board and the Regional Ethics Board, Sweden. All studies were conducted according to the Declaration of Helsinki ethical principles for medical research involving human subjects. The number of independent cases and control individuals from each population and the number of families are described in detail below and their clinical characteristics are summarised in Tables 5 and 6.

1.2 Finnish case-control cohort (Studies I-IV)

The Finnish case-control cohort consists of 177 DLE, 42 SCLE, 91 SLE (85 with DNA sample available) cases and 356 control individuals, and has been the basis for the genetic studies in this thesis. All patients with a clinical diagnosis of lupus attending the Departments of Dermatology of Helsinki or Tampere University Central Hospitals during 1995-2005 were identified from corresponding hospital registries and contacted by mail or phone. Patients visiting these hospitals for routine lupus controls in 2005-2006 were also asked to participate. The presence of LE-specific skin manifestations diagnosed by a dermatologist at some point of the disease course was a prerequisite for inclusion in the study. Correct diagnosis was verified from the patients' hospital records. The diagnosis of DLE was based on generally accepted clinical, histological and immunofluorescence findings and that of SCLE on the revised description (Sontheimer 2005). The diagnosis of SLE was based on the fulfilment of the 1982 revised ACR classification criteria (Tan *et al.* 1982), including the presence of systemic manifestations together with positive serology and skin manifestations, and was always verified by a rheumatologist. To confirm the correct diagnosis, a skin biopsy was taken from 294/310 (95%) of the patients. The diagnosis was based on clinical and laboratory findings in those patients from which the biopsy was not obtained, because of the lack of skin lesions at the time of investigation (especially in SLE), or because the biopsy was inconclusive. The participating patients were clinically examined by three dermatologists (S. Koskenmies, T. Hasan and J. Panelius) and interviewed using a structured questionnaire. A total of 356 anonymous control individuals (49% women) comprised unaffected spouses or common-law spouses of patients and a collection of unrelated healthy individuals. The clinical characteristics of the patients are summarised in Table 5, while a more comprehensive description of the patients is found in Study I (Koskenmies *et al.* 2008).

Table 5. Clinical characteristics of the Finnish patient cohorts used in this study. Percentage (%) of patients with each phenotype is shown.

	DLE (n=177)	SCLE (n=42)	SLE (n=85)	SLE families (n=236 patients)
Females	76	91	93	94
Mean age at onset (range)	42 (15-77)	54 (14-89)	36 (8-85)	29 (1-66)
Mean age at diagnosis (range)	45 (17-77)	56 (15-89)	40 (13-86)	33 (6-72)
Butterfly rash	11	17	72	51
Discoid rash	92	10	44	10
Annular SCLE lesions	1	43	12	na
Psoriasiform SCLE lesions	1	63	22	na
Photosensitivity	65	81	80	69
Mouth ulcers	4	0	18	18
Arthritis	2	7	66	83
Renal involvement	1	0	20	30
Leukopaenia	7	14	37	68
Thrombocytopaenia	5	5	17	16
Elevated antinuclear antibodies	28	79	98	na
Ro/SSA antibody positivity[1]	25	81	66	na
La/SSB antibody positivity[1]	4	39	31	na
Double-stranded DNA antibody positivity	9	5	37	na

[1]Because of differences in laboratory methods and reference values, only patients from Helsinki were included.

1.3 Finnish family cohort (Study III)

The Finnish family cohort includes 192 families, with a total of 236 SLE patients and their unaffected relatives. According to the estimated prevalence of SLE in Finland (Helve 1985), 80-85% of patients requiring hospitalisation were contacted at the time of recruitment, which began in 1995. Patients with a positive family history of SLE and fulfilling the ACR classification criteria (Tan *et al.* 1982) were asked to participate in the study together with their unaffected and/or affected family members. All subjects were interviewed by the same rheumatologist (H. Julkunen) and their medical records were reviewed. Blood samples were obtained from a total of 252 families, of which 53 were multiply affected by SLE and the remaining were families of sporadic patients. A proband from each family was analysed in combination with sporadic SLE cases from the Finnish case-control cohort in Study III (Hellquist *et al.* 2009). The clinical characteristics of the patients are described in Table 5 and more detailed information is given by Koskenmies *et al.* (2001).

1.4 Swedish case-control cohort (Study IV)

The Swedish case-control cohort is a large collection of patients with different connective tissue diseases and has been used for replication in Study IV. Swedish patients participating in a study assessing the incidence and prevalence of SCLE in Stockholm in 1996-2002 reported their occurrence of photosensitivity and skin symptoms in a questionnaire. Patients were examined clinically (K. Popovic and F. Nyberg) at the Department of Dermatology, Danderyd Hospital, Stockholm, and clinical data were completed based on patient history and medical records. The diagnoses of DLE and SCLE were based on clinical and histological features and those of SLE, SS and undifferentiated connective tissue disease were based on generally accepted criteria (Mosca *et al.* 1999; Tan *et al.* 1982; Vitali *et al.* 2002). The collection of this material has been recently described in detail (Popovic *et al.* 2007b).

Another Swedish sample set, consisting of patients with SS fulfilling the revised European criteria for diagnosis (Vitali *et al.* 2002) and attending the Karolinska University Hospital, Stockholm in 1998-2008, was also used for replication. The diagnosis was verified by a combination of clinical examination by rheumatologists or medical doctors and a questionnaire. In total, there were 164 Swedish patients, out of which 134 were positive for Ro/SSA autoantibodies that were detected as described (Popovic *et al.* 2007b; Tengner *et al.* 1998). The clinical characteristics of the Swedish patients are presented in Table 6. The population-based 295 control individuals (90.5% women) consisted of samples from the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study (Stolt *et al.* 2003).

Table 6. Clinical characteristics of the Swedish patient cohorts used in this study. Percentage (%) of patients with each phenotype is shown.

	Connective tissue disease patients (n=91)[1]	Sjögren's syndrome patients (n=73)[2]
Female	91	95
Mean age at onset (range)	58 (18-83)[3]	na
Mean age at diagnosis (range)	na	52 (20-87)
Butterfly rash	13	na
Discoid rash	19	na
Annular SCLE lesions	14	na
Psoriasiform SCLE lesions	0	na
Photosensitivity	100	na
Mouth ulcers	na	na
Arthritis	5	23
Renal involvement	5	4
Leukopaenia	na	18
Thrombocytopaenia	na	3
Elevated antinuclear antibodies	74	55
Ro/SSA antibody positivity	100	59
La/SSB antibody positivity	38	38
Double-stranded DNA antibody positivity	13	na

[1]Ro/SSA positive patients without signs of systemic inflammation=21, DLE=2, SCLE=8, SLE=31, SS=23, undifferentiated connective tissue disease=6 from the study of Popovic *et al.* (2007b)
[2]SS patients from Prof. Marie Wahren-Herlenius, Dept. of Medicine, Rheumatology Unit, Karolinska Institutet, Solna, Sweden
[3]Mean age at initial testing for Ro/SSA autoantibodies (Popovic *et al.* 2007b)
Na = data not available

1.5 Tissue samples (Studies II and V)

All tissue samples were archival formalin-fixed, paraffin-embedded skin sections obtained from the Department of Dermatology and HUSLAB, Helsinki University Central Hospital. The diagnoses were based on clinical and laboratory data (S. Koskenmies) and confirmed histologically by an experienced dermatopathologist (L. Jeskanen).

Study II In order to study TYK2 expression in skin, lesional biopsies were obtained from 25 patients, of whom seven had DLE, seven SCLE and 11 SLE. Tissue sections from normal skin (n=5), kidney (n=4), prostate cancer (n=3) and squamous cell carcinoma (n=3) were selected as positive controls based on experimental evidence (Ide *et al.* 2008; Nishio *et al.* 2001) and expression profile (NCBI UniGene; http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene). A total of 22 patients, of whom eight had DLE, six SCLE and eight SLE were used to investigate the cutaneous expression of IRF5. Tissue sections from normal skin (n=3), placenta (n=2), breast cancer (n=2) and lymph nodes (n=2) were selected as positive controls based on *IRF5* gene expression profile (NCBI UniGene).

Study V The tissue expression of MMP-3, -7, -9, -10, -14, -19 and -26 as well as their endogenous inhibitors TIMP-1 and -3 was examined in a total of 57 patients (58 samples altogether), of whom 20 had DLE, 17 SCLE and 20 SLE. To confirm that the expression of MMPs studied was not induced by mere UVA or UVB radiation without evidence of lupus, photoprovocated skin samples obtained from healthy volunteers 3-5 days after the last repeated provocation with 100 J UVA or with 1.5 minimal erythema dose of UVB (Saarialho-Kere *et al.* 1999) were included.

2 Methods

The methods used in this study have been described in detail in the original publications (Table 7). Some of the methods are presented in more detail below.

Table 7. Methods used in this thesis.

Method	Kit / Manufacturer / Reference	Study
Laboratory procedures		
DNA extraction	Flexigene DNA kit, Qiagen GmbH Hilden, Germany Puregene blood kit, Gentra Sytems Inc., Minneapolis, MN, USA (Lahiri and Nurnberger 1991; Miller *et al.* 1988)	II, III, IV
Immunohistochemistry	StreptABComplex/HRP Duet, Mouse/Rabbit, DakoCytomation, Glostrup, Denmark Vectastain Elite ABC kit, Vector laboratories, Burlingame, CA, USA (Saarialho-Kere *et al.* 1993)	II, V
TUNEL method	ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, Chemicon International, Temecula, CA, USA (Airola *et al.* 1997)	V
Histopathological analysis	(Jerdan *et al.* 1990) Investigator L. Jeskanen	V
Sequenom iPLEX SNP genotyping	Sequenom Inc., San Diego, CA, USA http://www.sequenom.com (Jurinke *et al.* 2002)	II, III, IV
PCR assay design	SpectroDESIGNER, Sequenom Inc.	II, III, IV
Extension reaction cleaning	SpectroCLEANER, Sequenom Inc.	II, III, IV
Microsatellite genotyping	(Dideberg *et al.* 2007; Koskenmies *et al.* 2004)	unpublished data
Analysis programs		
MassARRAY mass spectrometer	Bruker Daltonik GmbH, Bremen, Germany	II, III, IV
SpectroTYPER RT v. 3.3.0	Sequenom Inc.	II, III, IV
Molecular Dynamics MegaBACE™ 1000	Global Medical Instrumentation, Albertville, MN, USA	unpublished data
MegaBACE™ Genetic Profiler v. 2.0	GE Healthcare, Waukesha, WI, USA	unpublished data

Method	Kit / manufacturer / reference	Study
Statistical softwares		
PedCheck	(O'Connell and Weeks 1998)	III
Haploview v. 4.0	(Barrett *et al.* 2005)	II, III, IV, unpublished data
SNPHAP v. 1.3	(Clayton 2002) http://www-gene.cimr.cam.ac.uk/clayton/software/	II
SPSS v. 12.01, 13.0, 15.0	SPSS Inc., Chigago, IL, USA	I, II, II, IV, V
STATA v. 8.0	Stata, College Station, TX, USA (Melen *et al.* 2006)	III
VassarStats	Richard Lowry, Poughkeepsie, NY, USA http://faculty.vassar.edu/lowry/VassarStats.html	V
GraphPad Prism v. 4.03	GraphPad Sotfware, La Jolla, CA, USA	II, III, IV, unpublished data
Power for Association With Error program v. 1.2	(Gordon *et al.* 2002; Gordon *et al.* 2003) http://linkage.rockefeller.edu/pawe/	II, III, IV, unpublished data

2.1 Data storage and management

Blood vials and DNA tubes were equipped with corresponding sample identification numbers. No one other than the immediate local project members had access to the personal identification code. At the genotyping core facility an automated sample processing and tracking software platform in a laboratory information management system (Nautilus LIMS) was used. Samples were tracked using barcode labels and nonpersonally identifiable sample tracking numbers. The genotype data and information on affection status and clinical features were stored in file formats compatible with analysis software. The following investigators had routine access to different data sets: T. Järvinen, A. Hellquist, S. Koskenmies and P. Onkamo (clinical information only).

2.2 DNA extraction (Studies II-IV)

Blood was collected in ethylenediaminetetraacetic acid (EDTA) vials. Genomic DNA of the sporadic patients and control individuals was extracted from whole-blood samples by standard nonenzymatic methods using commercial kits according to manufacturer's instructions. DNA of the SLE families was extracted using the protocol by Lahiri and Nurnberger (1991). The traditional salting out method (Miller *et al.* 1988) was employed for Swedish samples.

2.3 Marker selection (Studies II-IV and unpublished data)

Studies II and III After careful review of SLE literature published prior to GWASs, a total of 20 SNPs (Appendix Table 1) were selected for genotyping in the following genes: *IRF5*, *TYK2*, *CRP*, *FCGR2A*, *CTLA4*, *PDCD1*, *GIMAP5*, *NOD2*, *PTPN22* and *TNF-α*. All these genes and their respective SNPs have shown either significant linkage or association with SLE in previous studies (De Jager *et al.* 2006; Duerr *et al.* 2006; Graham *et al.* 2006; Graham *et al.* 2007; Graham *et al.* 2007; Hellquist *et al.* 2007; Karassa *et al.* 2002; Kaufman *et al.* 2006; Prokunina *et al.* 2002; Russell *et al.* 2004; Sigurdsson *et al.* 2005; Suarez *et al.* 2005; van Heel *et al.* 2002). A subset of Finnish family material, consisting of 109 SLE patients and 121 unaffected pedigree members, was studied previously for association to *IRF5* and *TYK2* (Sigurdsson *et al.* 2005), but with partly different markers. Thus these genes could be considered positive controls for association in extended SLE sample set in Study III.

Out of the selected markers, rs2004640 in *IRF5*, rs1205 in *CRP*, rs2066843 in *NOD2* and rs2476601 in *PTPN22* failed to meet our genotyping quality criteria and were thus excluded from further analyses. The *NOD2* SNP rs2066845 was found to be monomorphic (MAF <0.5%) resulting in exclusion of this marker and the remaining *NOD2* marker rs2076756 was genotyped only in families. The assay design for the polymorphisms in *TNF-α* (rs1800629 and rs1800630) failed because of too many adjacent SNPs and therefore these polymorphisms were not included in the genotyping. Thorough investigation of *GIMAP5* markers has been recently conducted in an independent study of the family material (Hellquist *et al.* 2007).

Study IV Eleven SNPs (Appendix Table 2) within the *ITGAM* region showing replicated and robust association to SLE across several populations were selected for genotyping based on published results (Harley *et al.* 2008; Hom *et al.* 2008; Nath *et al.* 2008). The markers rs6565227 and rs1143678 had a success rate below a study inclusion threshold (<85%) and were thus excluded.

Unpublished study Altogether 37 additional SNPs, and one indel polymorphism in *IRF5-TNPO3* (Appendix Table 2) were genotyped alongside the *ITGAM-ITGAX* locus in lupus patients with cutaneous manifestations. Priority was given to genes and loci showing the strongest evidence of association with SLE in recently published GWASs: *STAT4, BANK1, TNFAIP3, IRF-TNPO3, BLK-FAM167A, KIAA1542, 1q25.1* and *PXK* (Graham *et al.* 2008; Harley *et al.* 2008; Hom *et al.* 2008; Kozyrev *et al.* 2008). A panel of genes yielding a

suggestive association in one GWAS (Harley *et al.* 2008) was also included in the study (*ATG5*, *ICA1*, *LYN*, *XKR6* and *SCUBE1*). At each locus, SNPs providing the best association signal in previous GWASs together with those already known to be of functional relevance, or previously found strongly associated to SLE, were selected.

2.4 Statistical power analysis (Studies II-IV)

Power calculations to detect associations to studied risk variants were performed assuming reported risk parameters in the original studies (Han *et al.* 2009b; Sigurdsson *et al.* 2005; Sigurdsson *et al.* 2008a) at a significance level of 0.05. The power of an allelic test to detect association to *IRF5* with an OR of 1.45 was 92% in DLE, 49% in SCLE and 97% in SLE. Patients with DLE had a maximum power of 57% to identify association to *TYK2* (OR=1.40); the corresponding numbers for SCLE and SLE patients were 22% and 70%, respectively. The average power to detect association of *ITGAM* variants in different patient cohorts was 84% in DLE, 42% in SCLE, 91% in SLE and 80% in Swedish patients when a minor allele frequency of 0.118% in controls and an OR of 1.7 were used.

2.5 Genotyping (Studies II-IV and unpublished data)

In Studies II-IV, the Sequenom genotyping platform was employed. The Sequenom methodology utilises matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry for detection of resulting polymerase chain reaction (PCR) products. For allele-specific primer extension (Jurinke *et al.* 2002) the iPLEX protocol was chosen, in which up to 40 SNPs can be multiplexed in a single PCR. The genotyping amplification reactions were performed as described in detail in Hellquist *et al.* (2007) with small modifications. All PCR and extension assays were designed using SpectroDESIGNER software. Unincorporated dNTPs were dephosphorylated by addition of shrimp alkaline phosphatase enzyme to each sample. Extension reactions were then conducted together with low and high mass primers and the Mass EXTEND Reagents Kit before being cleaned using SpectroCLEANER. Desalted primer extension products were analysed by a MassARRAY mass spectrometer. The resulting mass spectra was analysed for peak identification using the SpectroTYPER RT for iPLEX assays. All genotypes were independently verified by two investigators. Genotyping was performed as a core service at the Mutation Analysis Facility, Karolinska Institutet, Huddinge, Sweden (http://www.maf.ki.se).

The quality control of the SNP genotyping assays was performed according to standard operating procedures at the genotyping core facility. Each genotyped assay was validated to assure genotyping consistency and quality by comparing genotype concordance from the genotyping on our platform in a set of 14 CEPH trios (Centre d'Etude Polymorphisme Humaine, Utah residents with ancestry from northern and western Europe) with genotypes available through the HapMap consortium (http://www.hapmap.org). Internal concordance was analysed on 14 additional unrelated individuals of Caucasian descent. Hardy-Weinberg equilibrium (HWE) was analysed in a total of 55 unrelated individuals to ensure that each marker was in equilibrium ($P>0.05$). Furthermore, the percentage of negative controls (H_2O

samples) with genotypes for each assay was accounted for, where assays showing more than 50% positivity were excluded. Success rate of all assays was required to be ≥85%. Assays not fulfilling the quality criteria were excluded from further genotyping. In the following genotyping, 90 samples from each individual sample set were genotyped twice and analysed for concordance to assure genotyping consistency. In addition to analysing HWE, negative controls with genotypes and success rate as in validation step, PedCheck (O'Connell and Weeks 1998) was used to detect Mendelian inconsistencies in the family material and markers showing >10% inheritance errors were excluded.

2.6 Association analyses (Studies II-V and unpublished data)

Haploview program (Barrett *et al.* 2005) was used to explore descriptive genotyping data. Allele and haplotype frequencies were calculated, and LD patterns assessed through pairwise D' and r^2 values. In addition to Haploview, SNPHAP software (Clayton 2002) was used to construct individual *CTLA4* haplotypes for DLE patients (Study II). Allele and haplotype associations were investigated using a chi-squared (χ^2) test as implemented in Haploview and two-sided P-values reported. ORs with corresponding 95% confidence intervals were estimated. In Studies II and IV, permutation tests (10,000 iterations) were performed for correction of multiple testing. Study III was a replication study, in which nominal P-values less than 0.05 were considered significant, and empirical P-value to confirm observed associations was obtained by the permutation approach (10,000 iterations).

2.7 Statistical analysis (Studies I-V)

Study I To create an overall picture of differences in clinical features, autoimmune diseases and laboratory abnormalities between different LE subtypes or the sexes, several types of comparisons were made. Group frequencies of binary and categorical traits were compared by a χ^2 test or Fisher's exact test, where the asymptotical properties for the χ^2 test were not appropriate. In addition, comparisons to population frequencies of smoking and RA were performed by nonparametric binomial test. Between-group comparisons of continuous and normally distributed variables were compared by *t*-test and one-way ANOVA accompanied by post hoc tests, where an overall difference had been found. The Mann-Whitney *U*-test (comparisons between two independent groups) and Kruskall-Wallis test (comparison between more than two independent groups) were used for normal traits. Because of the exploratory nature of Study I, correction for multiple testing was only applied in the analysis of variance post hoc tests, where Sheffe's method was used as implemented in the SPSS statistical package. Patients with missing values were excluded from frequency tables, frequency and mean tests. Nominal P-values under 0.05 were considered significant.

Clustering analysis was employed to divide patients computationally into groups based on similarities in various clinical manifestations and laboratory findings (Table 8) without knowing their original diagnosis of LE subtypes. Patients from Tampere University Hospital were excluded from analysis because of missing data for Ro/SSA and La/SSB autoantibodies, and four additional patients were left out due to incomplete data in some variables. Clustering methods are exploratory tools designed to reveal natural clusters or groupings within a data set that would otherwise not be apparent. In Study I, the grouping was made on the basis of similarities and differences in the clinical data and other features of the patients, where the most similar individuals form the clusters. The SPSS Two-Step algorithm was applied to the clinical data. The actual diagnoses were intentionally left out to obtain the natural grouping of the disease subtypes. The Two-Step algorithm is able to handle both categorical and standardised continuous variables, and the optimal number of clusters is selected automatically by comparing the values of a model-choice criterion across different clustering solutions. The grouping is based on the similarity between the individuals in the array of features; the similarity, however, is evaluated with a log-likelihood method. The likelihood measure places a probability distribution on the variables. Continuous variables are assumed to be normally distributed, whereas categorical variables are assumed to be multinomial. All variables are assumed to be independent.

Study III A multiple logistic regression model was used to estimate the gene-gene interaction between *IRF5* and *TYK2* SNPs in SLE patients by adding an interaction term between the genotypes of interest, as described previously (Melen *et al.* 2006). A model-free coding with variables for common homozygotes, heterozygotes and rare homozygotes was used as a first approach. For SNPs with too few rare homozygotes, dominant coding was used. P-values were obtained by likelihood-ratio tests that measure the null-hypothesis of no interaction between the models with and without interaction term. Observed P-values estimate a departure from a multiplicative interaction model on the OR scale indicating whether the effect (OR) of one genotype is altered by the effects of another genotype.

Sudy V Chi-squared test or Fisher's exact test, when appropriate, were employed to compare the number of MMP-positive samples and histological parameters in different diagnostic groups. The Mann-Whitney *U*-test and Kruskall-Wallis test were used for comparing the severity of histological parameters between diagnostic groups.

Table 8. Clinical variables used in cluster analysis and to study association to *IRF5*, *TYK2* and *ITGAM*.

Clinical variable	Study I	Study II	Study III[2]	Study IV[3]
Sex	✓			
Malar rash	✓			✓
Disseminated ACLE lesions	✓			
DLE lesions	✓			✓
Annular or psoriasiform SCLE lesions	✓			
Other LE-nonspecific cutaneous manifestations	✓			
Self-reported photosensitivity	✓	✓	✓	✓
Arthritis	✓		✓	
Serositis	✓		✓	
Renal involvement			✓	✓
Neuropsychological symptoms	✓			
General symptoms	✓			
Raynaud's phenomenon	✓			
Sjögren's syndrome	✓			
Anaemia	✓	✓	✓	
Leucopaenia	✓	✓	✓	✓
Lymphopaenia	✓	✓	✓	✓
Thrombocytopaenia	✓	✓	✓	✓
Hypersedimentation	✓			✓
Complement 3 or 4 level under reference value	✓	C4	C4	✓
Elevated ANA or ENA antibody titres	✓	✓	✓	ANA
Positive RNP, Ro/SSA, La/SSB, dsDNA[1]	✓	Ro/SSA, dsDNA	Ro/SSA, dsDNA	dsDNA
Use of oestrogen	✓			
Smoking	✓			✓
Age at onset	✓			
Number of positive ACR criteria	✓			

[1]Ro/SSA and La/SSB information was not available for patients from Tampere UniversityHospital, and probands from SLE families.
[2]Family probands had imperfect data on complement 4 values, anaemia and serositis.
[3]Information was not available on smoking, hypersedimentation and complement levels for probands from SLE families.

Subphenotype analysis (Studies II-IV) The association of *IRF5*, *TYK2* and *ITGAM* risk variants with clinical characteristics was investigated by stratifying patients into subgroups according to the presence or absence of the manifestations listed in Table 8. In Study II, DLE patients were further grouped into carriers and noncarriers of the high risk *CTLA4* haplotype GGCGA in order to study whether these two groups differ clinically. The variables selected for analysis were those that differentiated the patients most by clustering method in the descriptive clinical study of our patient cohort (Study I; Koskenmies *et al.* 2008) or have been reported to show association (Baechler *et al.* 2003; Namjou *et al.* 2002b). Ro/SSA autoantibody information was not available for patients from Tampere University Hospital and SLE families; neither had the family probands data available on the following traits: complement concentrations, anaemia, serositis, smoking at disease onset and hypersedimentation.

The genotype (*IRF5*, *TYK2*, *ITGAM*) or haplotype (*CTLA4*) frequencies in cases with and without the certain feature were compared. For categorical variables, the significance of individual genotypes or haplotypes was assessed by χ^2, Fisher exact test or logistic regression. For continuous variables, two-way ANOVA was used. In Studies II and III, two-tailed P-values less than 0.05 after Bonferroni multiple testing correction were considered significant. In Study IV, two-tailed P-values less than 0.05 were considered significant without correction for multiple testing, since the clinical variables are known to correlate and adjustment would likely be overly conservative (Hom *et al.* 2008).

2.8 Immunohistochemistry (Studies II and V)

Immunohistochemical analyses were performed to study the expression of multiple MMPs as well as TIMPs, TYK2 and IRF5 proteins (Table 9) in involved and healthy skin as well as other relevant tissues. Immunostaining of tissue samples was carried out using the enzymatic avidin-biotin-peroxidase complex technique, in which biotinylated secondary antibody interacts with a complex of avidin-biotin peroxidase bound to primary antibody. Diaminobenzidine (DAB), aminoethylcarbazole or NovaRED peroxidase (Vector laboratories, Burlingame, CA, USA) were used as chromogenic substrates and Mayer's hematoxylin as counterstain, as described in detail elsewhere (Saarialho-Kere *et al.* 1993). Following standard deparaffinisation by xylene treatment and decreasing alcohol series, endogeneous peroxidase activity was blocked by incubation with 3% H_2O_2 for 10 min in PBS at room temperature. Sections were pretreated for 10-30 min when necessary. Primary mono- or polyclonal antibodies (Table 9) were applied on slides and incubated for 45 min - 2 hours at 37°C or overnight at 4°C in a humified chamber. Control experiments were performed using rabbit and mouse preimmune serum as primary antibodies at equivalent dilutions (Table 9). Immunohistochemical specimens were analysed by four different investigators (T. Järvinen, P. Kanninen, L. Jeskanen and U. Saarialho-Kere) under a light microscope at 200x magnification. Semiquantitative immunoreactivity grading was based on positive staining in the epidermis and dermis and scored as follows: 0<10 positive cells, 1=10-20 positive cells, 2=20-50 positive cells and 3>50 positive cells.

Table 9. Antibodies used in immunohistochemistry.

Antibody	Source	Dilution	Pretreatment
MMP-3	ab32607, Abcam	1:85	+95°C water bath in citrate buffer
MMP-7	IM40L, Calbiochem	1:100	+95°C water bath in citrate buffer
MMP-9	MS-569, LabVision	1:75	Trypsin 10mg/ml
MMP-10	NCL-MMP10, Novocastra laboratories	1:300	Trypsin 10 mg/ml
MMP-14	MT1-MMP (current RB-1544), LabVision	1:400	No pretreatment
MMP-19	PC374, Oncogne	1:45	No pretreatment
MMP-26	Prof. Keiichi Isaka (Ahokas *et al.* 2005)	1:150	+95°C water bath in citrate buffer
TIMP-1	IM63, Calbiochem	1:100	+95°C water bath in citrate buffer
TIMP-3	IM43L, Calbiochem	1:500	+95°C water bath in citrate buffer
TYK2	sc-169, SantaCruz Biotechnology	1:500	+95°C water bath in citrate buffer
IRF5	ab55336, Abcam	3 µg/ml	+95°C water bath in citrate buffer

RESULTS

1 Smoking characterises DLE (Study I)

Altogether three hundred and ten DLE, SCLE and SLE patients with LE-specific cutaneous manifestations were recruited using patients' charts from institutional databases and during routine control visits. Clinical data collected from the medical records included the 11 ACR criteria completed by more detailed information on the cutaneous and general symptoms and the year of diagnosis. Salient clinical manifestations and laboratory findings are summarised below, while more detailed information is presented in Study I and Tables 2-6 therein (Koskenmies *et al.* 2008).

The mean age (standard deviation) at onset was 42 (15) years in DLE, 55 (19) years in SCLE and 31 (16) years in SLE and the difference between patient groups was significant (P=0.001). Patients with DLE and SCLE were characterised with typical discoid, annular or psoriformic lesions, whereas malar rash, oral ulcerations and disseminated ACLE lesions were most often seen in SLE patients. Only a few patients with DLE also had ACLE lesions and one third of SCLE patients had malar rash. Photosensitivity was self-reported in most cases, and characterised all groups, in particular SCLE patients, of whom 87% reported sun-induced LE rash or worsening of existing LE rash in sunlight. Provocative phototesting (either UVA and/or UVB) was positive in 21% (8/38) of tested patients. Raynaud's phenomenon was the most frequent nonspecific LE skin manifestation observed and typically concurrent with SLE, whereas other nonspecific manifestations (vasculitis, alopecia, urticaria and livedo reticularis) were rare in all groups.

In general, laboratory value abnormalities were most often detected in patients with SLE. Interestingly, lymphopaenia and hypersedimentation were common in all patients, although with higher frequencies in SCLE (44% and 56%, respectively) and SLE (52% and 71%, respectively) patients. As expected, patients with DLE and SCLE had less immunological defects compared to SLE patients. However, ENA autoantibodies and especially Ro/SSA autoanbodies showed elevated titre levels more often in SCLE (83%) than in SLE (62%) patients, while less than 30% of DLE patients were positive for ENA autoantibodies. In contrast, ANAs were detected slightly more frequently among SLE (96%) than in SCLE (84%) cases. Association of positive ENA and ANA tests with certain clinical features was further investigated. There was a tendency of Ro/SSA and La/SSB autoantibody positive patients to report photosensitivity (P=0.03) and ANA positivity coincided with malar rash, disseminated ACLE lesions and cutaneous manifestations of SCLE.

Systemic manifestations are rarely seen in other patients with LE than those with SLE, in which arthritis (68%) was the most commonly observed. Three patients with renal involvement and one with serositis were found in the DLE group, while none of the SCLE patients presented with these defects. Approximately one fourth of SLE patients had renal involvement and serositis. Other abnormalities related to kidney malfunction, such as

proteinuria and haematuria, were rare among DLE and SCLE cases, whereas around 20% of SLE cases had proteinuria or haematuria at some point of their disease course. Neurological findings were rare in all patient groups.

Personal history of autoimmune disease other than LE was more often reported by SCLE and SLE than DLE patients. Of the concomitant autoimmune diseases, SS was the most common followed by autoimmune thyroid disease in all subgroups. However, these diseases were observed at substantially higher frequencies in SCLE (16% and 13%, respectively) and SLE (22% and 11%, respectively) than in patients with DLE (2% and 3%, respectively). Interestingly, the prevalence of RA seemed to be increased among SCLE patients (6%). However, age/gender-matched comparison showed no difference between cases and the background population, which has a prevalence of 1% for RA (Hakala *et al.* 1993).

High frequency of smoking was characteristic to all patient groups. At the time of first symptoms, 57% of DLE, 35% of SCLE and 34% of SLE patients smoked daily. Notably, as many as 51% of women with DLE were smokers indicating that the high proportion of smoking in this subgroup is not due to male predominance (62% were smokers). Smoking was more prevalent in LE patients compared with the population in general: 63% of female and 57% of male patients at age 25-44 years smoked daily, while the corresponding numbers were 22% and 35%, respectively, in the age/gender-matched background population (Statistics Finland 2005).

Clustering analysis showed three separate clusters not entirely coinciding with clinical subgroups (Figure 4). When the cluster-membership of the patients was cross-checked against diagnoses, it was found that cluster 3 was an equal combination of patients with DLE and SCLE (35 and 29, respectively), whereas cluster 1 represented SLE patients (46/56 members) and cluster 2 consisted of DLE patients (99/102 members). Of particular interest is the observation that daily smoking was one of the characteristics that differentiated cluster 2 from other clusters. Other important differences between the clusters were found in variables such as elevated titre levels of ENA (especially Ro/SSA autoantibodies) and ANA, malar rash, discoid lesions, disseminated ACLE lesions, serositis, Raynaud's phenomenon, arthritis, low serum complement 3 and 4 concentrations, and a number of ACR criteria. Based on the results, haematological and immunological findings seemed to account more for cluster formation than did conventionally used histological and immunological features.

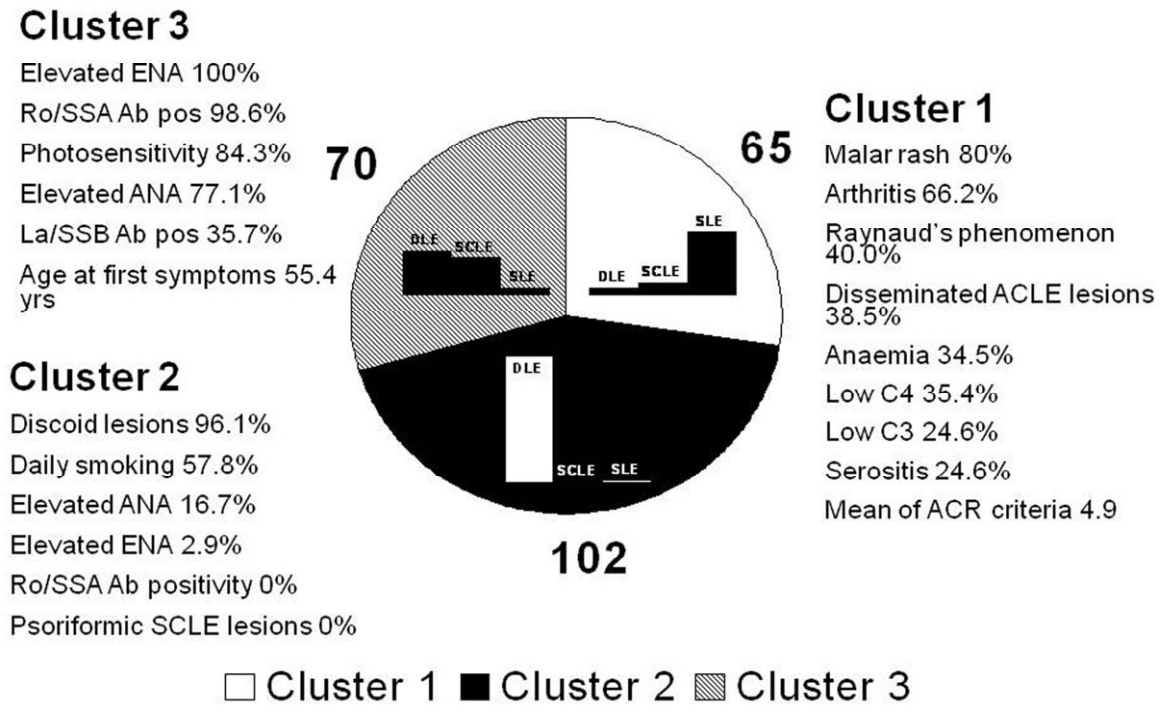

Figure 4. The cluster sizes and the most important differentiating variables with their representative frequencies for each cluster. The mean of American College of Rheumatology criteria was 4.9, 2.4 and 3.0 for clusters 1, 2 and 3, respectively.

2 *IRF5* and *TYK2* confer risk for DLE and SCLE (Study II)

A panel of 15 SNPs in six genes implicated previously in SLE pathogenesis was successfully genotyped in a total of 219 patients with DLE or SCLE and their respective control individuals. *IRF5*, *TYK2* and *CTLA4* were further investigated for association with relevant clinical characteristics (Table 8). In addition, the expression of IRF5 and TYK2 proteins in lesional skin was studied employing immunohistochemistry.

Single allele association results in DLE and SCLE patients are presented in Table 10. DLE and SCLE patients carrying the A allele of *IRF5* marker rs10954213 had increased disease risk compared to controls (OR=1.40, 95% CI=1.06-1.86 and OR=1.87, 95% CI=1.09-3.21, respectively). *TYK2* showed association only to DLE, and the lack of association in SCLE may reflect the modest size of the particular patient cohort. Significant association was observed in DLE patients carrying the C allele of *TYK2* marker rs2304256 (adjusted P=0.04), while two other markers studied did not reach significant association. However, on the haplotype level, the GCA allele combination of three genotyped *TYK2* markers was found to be associated with increased risk of DLE (P=0.05, OR=1.43, 95% CI=1.07-2.0). None of the individual SNPs in *CTLA4* showed disease association to CLE, but GGCGA haplotype

demonstrated highly increased risk in patients with DLE (P=0.03, OR=2.51, 95% CI=1.25-5.04).

Other genes studied, namely *FCGR2A*, *PDCD1* and *GIMAP5*, did not show association with CLE. Interestingly, the A allele of rs1801274 in *FCGR2A* was abundant among patients with DLE, whereas in contrast, SCLE patients were carriers of the G allele. The G allele of rs11568821 in *PDCD1* was slightly more common in patients than in controls. In line with our previous report on Finnish SLE trios (Hellquist *et al.* 2007), the most frequent *GIMAP5* haplotype among patients was CCGT (data not shown).

Table 10. Single allele and haplotype association results in DLE, SCLE and SLE patients.

		DLE (n=177)					SCLE (n=42)					SLE (n=277)				
Gene	**SNP ID**	**Associated allele / haplotype**	**Control freq**	**Case freq**	**P-value**	**OR (95% CI)**	**Associated allele / haplotype**	**Control freq**	**Case freq**	**P-value**	**OR (95% CI)**	**Associated allele / haplotype**	**Control freq**	**Case freq**	**P-value**	**OR (95% CI)**
FCGR2A	rs1801274	A	0.50	0.52	0.51		G	0.50	0.59	0.14		G	0.50	0.54	0.15	
CTLA4	rs231775	G	0.50	0.55	0.19		G	0.51	0.56	0.34		A	0.49	0.51	0.47	
CTLA4	rs3087243	A	0.35	0.36	0.61		G	0.66	0.71	0.37		G	0.66	0.66	0.87	
CTLA4	rs231726	T	0.46	0.47	0.81		T	0.46	0.54	0.19		C	0.54	0.56	0.53	
CTLA4	rs231727	A	0.46	0.47	0.80		A	0.46	0.54	0.19		G	0.54	0.56	0.58	
CTLA4	rs1991416	A	0.53	0.54	0.95		G	0.47	0.51	0.44		A	0.53	0.55	0.49	
CTLA4		GGCGA	0.02	0.05	**0.0065***	2.51 (1.25-5.04)										
PDCD1	rs11568821	G	0.92	0.94	0.26		G	0.92	0.95	0.39		C	0.92	0.95	0.06	
IRF5	rs10954213	A	0.62	0.69	**0.017***	1.40 (1.06-1.86)	A	0.62	0.75	**0.022**	1.87 (1.09-3.21)	A	0.62	0.70	**0.0043**	1.42 (1.12-1.81)
GIMAP5	rs759011	T	0.38	0.38	0.95		C	0.62	0.63	0.87						
GIMAP5	rs1046355	T	0.38	0.38	0.95		C	0.62	0.63	0.87						
GIMAP5	rs6598	G	0.62	0.62	0.82		G	0.61	0.64	0.76						
GIMAP5	rs2286899	T	0.74	0.76	0.61		T	0.74	0.79	0.34						
NOD2	rs2076756											A	0.85	0.84	0.44	
TYK2	rs12720270	G	0.78	0.81	0.20		A	0.22	0.26	0.41		G	0.78	0.85	**0.0031***	1.57 (1.16-2.21)
TYK2	rs2304256	C	0.69	0.76	**0.0117***	1.47 (1.09-1.98)	A	0.31	0.33	0.71		C	0.69	0.79	**0.0001***	1.68 (1.29-2.18)
TYK2	rs12720356	A	0.91	0.98	0.08		A	0.91	0.95	0.23		A	0.91	0.93	0.07	
TYK2		GCA	0.69	0.76	**0.0173***	1.43 (1.07-2.00)						GCA	0.69	0.78	**0.0002***	1.63 (1.26-2.12)
TYK2												AAA	0.22	0.15	**0.0030***	0.64 (0.47-0.86)

The frequency of the associated allele in controls and cases is shown, as well as its P-value and odds ratio (OR) with 95% confidence interval (CI). P-values in bold are statistically significant (P<0.05) and P-values marked with asterisk remained significant after correction for multiple testing.
FCGR2A, Fc fragment of IgG, low affinity IIa, receptor (CD32); *CTLA4*, cytotoxic T-lymphocyte-associated protein 4; *GIMAP5*, GTPase, IMAP family member 5; *IRF5*, interferon regulatory factor 5; *NOD2*, nucleotide-binding oligomerization domain containing 2; *PDCD1*, programmed cell death 1; *TYK2*, tyrosine kinase 2

Based on genetic association results, IRF5 and TYK2 were selected for subsequent immunohistochemical studies. The results are presented graphically in Figures 1-2 in Study II (Järvinen *et al.* 2010). IRF5 protein expression was observed in the upper keratinocyte layers of the epidermis in skin samples obtained from patients with CLE or SLE. Positive staining was observed in macrophage-like cells, fibroblast and plasma cells in the dermis, but neither in lymphocytes nor in endothelial cells. Normal skin specimens showed positivity in the granular and suprabasal layers of the epidermis. Positive immunoreactivity was found in plasma cells and macrophage-like cells in lymph nodes and in plasma cells and occasional cancer cells in breast cancer specimens. Lymph nodes with squamous cell carcinoma metastasis showed positive staining in a subpopulation of cancer cells. Control experiments with preimmune IgG did not result in any staining.

In skin samples obtained from patients with different subtypes of LE, TYK2 protein was detected in the granular layer of the epidermis and in suprabasal keratinocytes in all subgroups. Normal skin specimens taken from healthy controls showed positive staining in keratinocytes located in the granular and suprabasal layers. Staining intensity did not differ between diseased and normal skin. Within the infiltrating inflammatory cells of LE lesions, TYK2 was expressed by macrophage-like cells and neutrophils in the dermis. Fibroblast-like cells were also positive in some biopsies. Furthermore, kidney samples showed immunoreactivity in neutrophils. One third of squamous cell carcinoma samples showed positive staining in basal keratinocytes and occasional inflammatory cells, while no staining was seen in invading cancer cells. Control experiments with preimmune IgG did not result in any staining.

3 *IRF5* and *TYK2* show evidence of gene-gene interaction (Study III)

The SLE case-control material was genotyped in conjunction with SLE families, and a proband from each family was analysed together with sporadic cases to obtain maximal statistical power to detect associations. Six genes with published prior GWASs and suggested to be of importance in SLE susceptibility were studied, including *FCGR2A*, *CTLA4*, *PDCD1*, *IRF5*, *NOD2* and *TYK2*. The disease risk was further studied by estimating epistasis, that is, gene-gene interaction. Motivated by our own results (Koskenmies *et al.* 2008) and previous reports (Baechler *et al.* 2003; Namjou *et al.* 2002b), patients were stratified by various clinical features (Table 8) in order to explore genetic association to these parameters.

Consistent with the original report on SLE (Sigurdsson *et al.* 2005) and subsequent replication studies (Cunninghame Graham *et al.* 2007; Graham *et al.* 2007; Graham *et al.* 2006; Graham *et al.* 2007; Sigurdsson *et al.* 2008a), we were able to reproduce the association to *IRF5* and *TYK2* in an extended cohort of Finnish SLE patients (Table 10). In *IRF5*, the only successfully genotyped SNP rs10954213 showed significant association with the disease (P=0.0043, OR=1.42, 95% CI=1.12-1.81). Two out of three studied markers in *TYK2*, rs2304256 and rs12720270, reached the level of significant association (P=0.0001, OR=1.68, 95% CI=1.29-2.18 and P=0.0031, OR=1.57, 95% CI=1.16-2.21, respectively). Haplotype association test was accomplished for *TYK2* and *CTLA4*, of which the former showed

association to one risk (OR=1.63, 95% CI=1.26-2.12) and one protective haplotype (OR=0.64, 95% CI=0.47-0.86). None of the other genes tested demonstrated association to SLE.

Given that both *IRF5* and *TYK2* are members of the type 1 IFN pathway, a possible gene-gene interaction was studied between genotyped markers in these genes. Significant overall interaction could be observed between rs10954213 in *IRF5* and rs2304256 in *TYK2* (P=0.014). Figure 5 shows that the risk alleles for rs10954213 (AA) and rs230456 (CC) contributed most to the overall interaction (P<0.0001) whereas the remaining combinations were insignificant. A tendency towards interaction could be observed between rs12720270 and rs10954213 (P=0.096) and rs12720356 and rs10954213 (P=0.083).

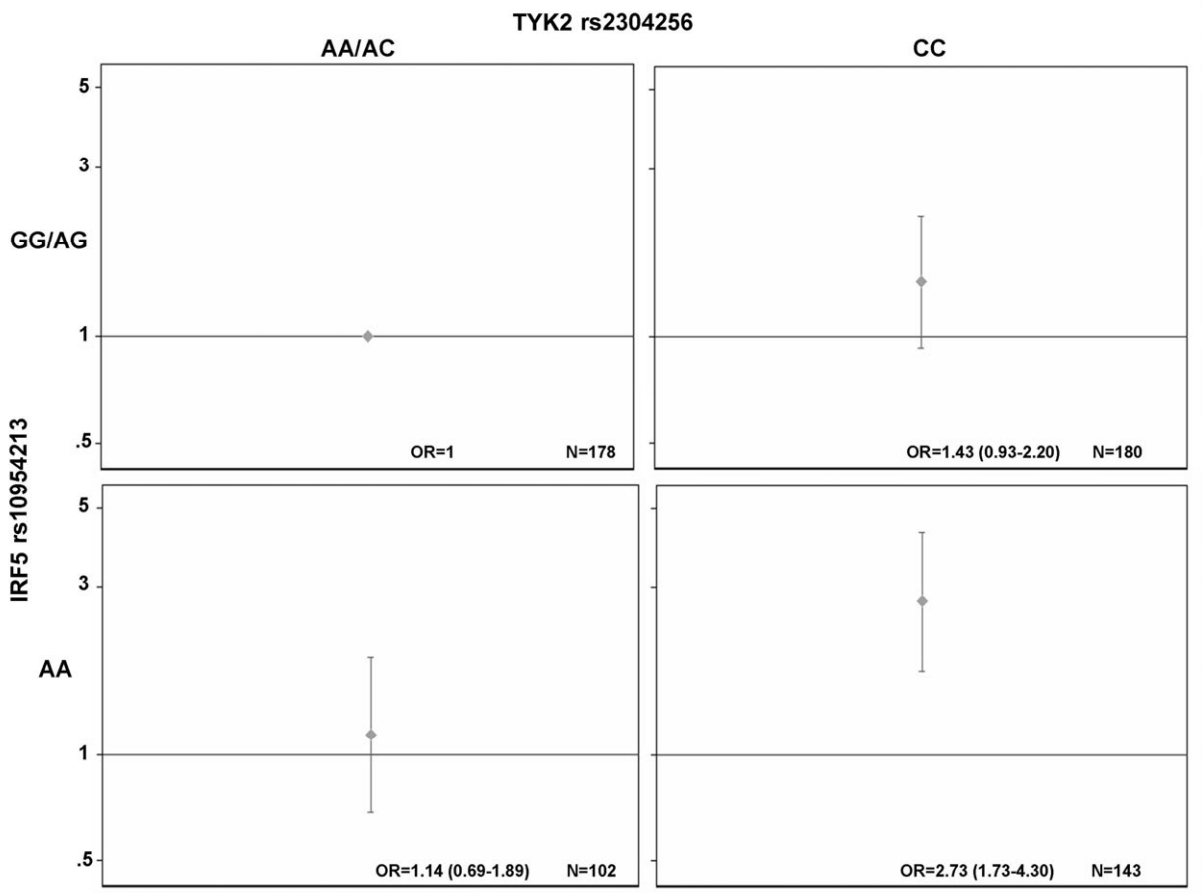

Figure 5. Genetic interaction between *TYK2* and *IRF5*. 2 x 2 table illustrates the interactive effect of *TYK2* rs2304256 and *IRF5* rs10954213 in SLE. Individuals homozygous for the rs2304256 risk allele C and the rs10954213 risk allele A show significantly increased risk effect compared to individuals homozygous or heterozygous for nonrisk alleles. OR (95% CI) for each combination and number of subjects (N) in each category are shown.

4 *ITGAM* is a novel susceptibility gene with high risk estimate for DLE (Study IV)

ITGAM was identified as a SLE susceptibility gene simultaneously in a traditional case-control study setting (Nath *et al.* 2008) and two GWASs (Harley *et al.* 2008; Hom *et al.* 2008). Several replication studies (Han *et al.* 2009b; Molineros *et al.* 2009; Yang *et al.* 2009) have been published thereafter, which encouraged us to investigate the role of this gene in the pathogenesis of CLE and compare disease risk to that of SLE. A cohort of Swedish patients was also included to study the association between *ITGAM* and Ro/SSA autoantibodies in order to replicate recent findings (Yang *et al.* 2009). Nine out of eleven SNPs were successfully genotyped (Appendix Table 2) in three Finnish datasets corresponding to two different LE subgroups (DLE and SCLE) and control individuals. Sporadic SLE patients from the case-control cohort were analysed together with probands from SLE families in an independent study (Hellquist *et al.*).

The most remarkable result in Study IV was the observation that studied polymorphisms associated with even higher risk for DLE (OR-range of 1.65-3.20) compared to that of SLE (OR-range of 1.52-2.14) (Table 11). The markers rs1143679, rs9888879 and rs11860650 contributed to the strongest association signal ($P=10^{-11}$) among patients with DLE. The same markers showed similar tendency in SCLE patients ($P=10^{-2}$). All other markers tested in DLE, except rs9937837, reached a P-value of $\leq 10^{-8}$ and an OR of ≥ 2.50. As expected, the strong association of *ITGAM* with SLE was replicated in Finnish patients ($10^{-3} \geq P \geq 10^{-6}$) (Hellquist *et al.*). A haplotype analysis was also performed in each set of samples, but this neither improved statistical significance nor added any further information, since most of the detected haplotypes were due to individual SNPs and their alleles as detected in single-marker analysis. The ATGCATTTC haplotype carrying allele A at rs1143679 accounted most for the risk in both DLE (OR=3.33), SCLE (OR=2.01) and SLE (OR=2.19) (see Study IV Supplementary material for details)

The association between *ITGAM* and Ro/SSA autoantibody positivity was further investigated. There were altogether 101 Finnish and 134 Swedish Ro-positive patients that were separately compared to healthy control individuals. The marker rs1143679 showed the strongest association in both cohorts and the observed ORs were overlapping in Finnish (OR-range of 1.40-2.65) and Swedish patients (OR-range of 1.16-1.80) (Table 12).

Previous reports show that LE and SS have shared susceptibility genes, namely *IRF5* and *STAT4* (Korman *et al.* 2008; Miceli-Richard *et al.* 2007; Nordmark *et al.* 2009). Based on the clinical survey in Study I, SS (30/310 patients) was the most common related autoimmune disease in patients with DLE, SCLE and SLE (Koskenmies *et al.* 2008). Moreover, a substantial proportion of the Swedish patients presented with SS (96/164; Table 6). Therefore, after power calculations had shown sufficient power (83%), it was tested whether *ITGAM* risk alleles appear with higher frequency in patients than in controls (Järvinen *et al.*, unpublished data). A modest association was found to markers rs1143679 (P=0.002), rs9936831 (P=0.008) and rs129288 (P=0.004) in patients with concurrent SS. Risk estimates (OR-range of 1.24-1.84) corresponded to those observed in Ro/SSA-positive patients (Table 12). Other tested

markers showed borderline or no association ($P \geq 10^{-2}$; data not shown). Similar to CLE, SLE (Hellquist *et al.*) and Ro-positive patients, haplotype analysis did not provide any further information. However, the same ATGCATTTC haplotype had a predisposing effect (P=0.003, OR=1.84, 95% CI=1.23-2.74) as in other studied cohorts.

Table 11. *ITGAM* allele association results in Finnish DLE, SCLE and SLE case-control materials.

Marker	Alleles[1]	Associated allele	Controls (n=356)	DLE (n=177)			SCLE (n=42)			SLE (n=275)[2]		
			Freq.	Freq.	P-value	OR (95% CI)	Freq.	P-value	OR (95% CI)	Freq.	P-value	OR (95% CI)
rs1143679	A/G	A	0.10	0.26	**4.73×10^{-11}**	3.20 (2.23-4.57)	0.19	0.02	2.17 (1.15-4.09)	0.19	**8.29×10^{-6}**	2.14 (1.52-3.00)
rs9936831	T/A	T	0.10	0.26	**2.14×10^{-10}**	3.03 (2.13-4.31)	0.18	0.06	1.86 (0.97-3.57)	0.19	**1.32×10^{-5}**	2.08 (1.49-2.90)
rs9937837	G/T	G	0.24	0.34	**6.00×10^{-4}**	1.65 (1.24-2.20)	0.26	0.62	1.14 (0.67-1.94)	0.32	**1.20×10^{-3}**	1.52 (1.18-1.96)
rs9888879	C/T	C	0.10	0.26	**7.57×10^{-11}**	3.06 (2.16-4.34)	0.18	0.05	1.84 (0.98-3.45)	0.19	**1.10×10^{-5}**	2.06 (1.49-2.86)
rs12928810	A/G	A	0.10	0.23	**8.03×10^{-8}**	2.77 (1.89-4.07)	0.15	0.14	1.67 (0.83-3.34)	na[3]	na	na
rs9888739	T/C	T	0.10	0.25	**2.91×10^{-10}**	3.01 (2.12-4.29)	0.18	0.04	1.88 (1.01-3.53)	0.18	**7.52×10^{-5}**	1.96 (1.40-2.74)
rs11860650	T/C	T	0.10	0.27	**3.71×10^{-11}**	3.13 (2.21-4.43)	0.18	0.04	1.94 (1.03-3.64)	0.19	**3.36×10^{-5}**	1.99 (1.43-2.77)
rs4548893	T/C	T	0.15	0.32	**1.10×10^{-10}**	2.74 (2.00-3.74)	0.21	0.12	1.58 (0.89-2.81)	0.22	**9.00×10^{-4}**	1.64 (1.22-2.21)
rs11574637	C/T	C	0.15	0.30	**2.37×10^{-9}**	2.57 (1.87-3.52)	0.21	0.11	1.58 (0.89-2.83)	0.22	**5.00×10^{-4}**	1.69 (1.25-2.27)

The frequency of the associated allele in controls and cases is shown, as well as its P-value and odds ratio (OR) with 95% confidence interval (CI). P-values in bold remain significant after correction for multiple testing.

[1]Minor allele / major allele

[2]From the study by Hellquist *et al.*

[3]The marker has a success rate below the study threshold (<85%) and was excluded from analysis in SLE.

Table 12. *ITGAM* locus showed evidence of association in Ro/SSA autoantibody positive Finnish and Swedish patients.

Marker	Alleles[1]	Associated allele	Finnish				Swedish			
			Controls (n=356)	Patients (n=101)			Controls (n=295)	Patients (n=134)		
			Freq.	Freq.	P-value	OR (95% CI)	Freq.	Freq.	P-value	OR (95% CI)
rs1143679	A/G	A	0.10	0.22	**6.61×10^{-6}**	2.65 (1.71-4.10)	0.10	0.15	0.04	1.62 (1.03-2.54)
rs9936831	T/A	T	0.10	0.23	**1.34×10^{-5}**	2.54 (1.65-3.90)	0.11	0.16	0.05	1.54 (0.99-2.39)
rs9937837	G/T	G	0.24	0.30	0.06	1.40 (0.99-2.00)	0.25	0.31	0.10	1.32 (0.95-1.83)
rs9888879	C/T	C	0.10	0.23	**6.31×10^{-6}**	2.55 (1.68-3.87)	0.11	0.15	0.05	1.55 (1.00-2.40)
rs12928810	A/G	A	0.10	0.18	**0.006**	1.96 (1.21-3.19)	0.10	0.16	0.01	1.80 (1.14-2.82)
rs9888739	T/C	T	0.10	0.22	**1.47×10^{-5}**	2.51 (1.64-3.85)	0.11	0.15	0.08	1.48 (0.96-2.31)
rs11860650	T/C	T	0.10	0.23	**9.86×10^{-6}**	2.54 (1.66-3.87)	0.11	0.14	0.17	1.37 (0.87-2.16)
rs4548893	T/C	T	0.15	0.27	**6.96×10^{-5}**	2.15 (1.46-3.14)	0.19	0.21	0.43	1.16 (0.80-1.68)
rs11574637	C/T	C	0.15	0.25	**4.00×10^{-4}**	1.99 (1.35-2.93)	0.16	0.21	0.08	1.39 (0.96-2.04)

The frequency of the associated allele in controls and cases is shown, as well as its P-value and odds ratio (OR) with 95% confidence interval (CI). P-values in bold remain significant after correction for multiple testing.

[1]Minor allele / major allele

5 Several of the GWAS-identified SLE susceptibility genes are replicated in lupus erythematosus patients with cutaneous manifestations (unpublished data)

Multiple new susceptibility genes were identified, and known candidates confirmed for SLE in recently conducted GWASs (Graham *et al.* 2008; Harley *et al.* 2008; Hom *et al.* 2008; Kozyrev *et al.* 2008). Thirteen genes (Appendix Table 2) with the strongest evidence of association were tested for disease susceptibility in 304 lupus patients with cutaneous manifestations. Genes *STAT4*, *BANK1*, *TNFAIP3*, *IRF5-TNPO3*, *BLK-FAM167A* and *1q25.1* locus showed significant associations both in DLE and SLE patients (Table 13) whereas genes *PXK*, *ATG5*, *ICA1*, *XKR*, *LYN*, *KIAA1542* and *SCUBE1* did not reach statistical significance.

All markers in *BLK-FAM167A* resulted in strong association to DLE (unadjusted P-values of 10^{-4} and 10^{-3}), while more modest results were seen in SLE ($P=10^{-2}$). *IRF5-TNPO3*, on the contrary, showed robust association ($10^{-1} \geq P \geq 10^{-7}$) to SLE whereas borderline association ($P=10^{-2}$) was demonstrated in DLE patients. *IRF5-TNPO3* was the only gene that associated with SCLE (P=0.04, OR=1.74, 95% CI=1.02-2.97 for rs729302 and P=0.003, OR=2.12, 95% CI=1.28-3.50 for CGGGG indel), possibly reflecting the insufficient sample size of the cohort (data not shown). *STAT4* and *TNFAIP3* showed similar patterns of association as *IRF5-TNPO3*. A marginal association was observed to *BANK1* and the *1q25.1* locus in patients with DLE. In terms of risk estimates, the highest ORs were detected for *TFNAIP3* in all LE subgroups, while other genes showed a smaller increase in disease risk. No improvement was found in obtained P-values after haplotype analyses were completed (data not shown).

Table 13. GWAS-identified genes associating with DLE and SLE patients with cutaneous manifestations. Significant P-values less than 0.05 are shown.

Gene	SNP	Associated allele	Controls (n=356)	DLE (n=177)			SLE (n=85)		
			Freq	Freq	P-value	OR (95% CI)	Freq	P-value	OR (95% CI)
1q25.1	rs10798269	G	0.68	0.74	0.03	1.39 (1.03-1.85)	0.72		1.23 (0.84-1.77)
STAT4	rs3821236	A	0.24	0.27		1.18 (0.88-1.60)	0.35	**0.004**	1.73 (1.19-2.50)
STAT4	rs7601754	G	0.11	0.13		1.28 (0.86-1.91)	0.11		1.05 (0.60-1.81)
STAT4	rs7574865	T	0.20	0.26	0.04	1.39 (1.01-1.88)	0.30	**0.006**	1.70 (1.16-2.49)
STAT4	rs10181656	G	0.20	0.25		1.33 (0.98-1.81)	0.30	**0.007**	1.68 (1.15-2.46)
STAT4	rs7582694	C	0.20	0.25		1.30 (0.95-1.77)	0.31	**0.005**	1.71 (1.17-2.50)
BANK1	rs10516487	G	0.68	0.74	0.03	1.39 (1.04-1.86)	0.69		1.08 (0.75-1.56)
TNFAIP3	rs6920220	A	0.17	0.24	**0.01**	1.52 (1.11-2.10)	0.24		1.49 (0.99-2.23)
TNFAIP3	rs10499197	G	0.01	0.03	0.04	2.50 (1.03-6.10)	0.05	**0.001**	4.18 (1.63-10.71)
TNFAIP3	rs5029939	G	0.02	0.03		2.01 (0.86-4.68)	0.05	**0.005**	3.38 (1.38-8.29)
TNFAIP3	rs2230926	G	0.02	0.03		2.21 (0.93-5.25)	0.05	**0.009**	3.32 (1.29-8.56)
TNFAIP3	rs7749323	A	0.02	0.03		2.01 (0.86-4.68)	0.04	0.04	2.66 (1.02-6.98)
IRF5-TNPO3	rs729302	A	0.62	0.67		3.23 (2.45-4.25)	0.83	**$3.8x10^{-7}$**	2.94 (1.91-4.52)
IRF5-TNPO3	CGGGG indel[1]	4x CGGGG	0.43	0.49		1.41 (1.05-1.88)	0.61	**$1.0x10^{-4}$**	2.08 (1.43-3.04)
IRF5-TNPO3	rs3807306	T	0.50	0.60	**0.002**	1.53 (1.18-2.01)	0.60	0.01	1.54 (1.09-2.18)
IRF5-TNPO3	rs2070197	C	0.15	0.21	0.02	1.48 (1.05-2.09)	0.25	**0.002**	1.92 (1.27-2.91)
IRF5-TNPO3	rs10488631	C	0.15	0.20	0.04	1.42 (1.01-2.01)	0.24	**0.003**	1.87 (1.23-2.85)
IRF5-TNPO3	rs2280714	T	0.71	0.77		1.32 (0.97-1.79)	0.76		1.29 (0.87-1.92)
IRF5-TNPO3	rs12539741	T	0.15	0.21	0.03	1.46 (1.04-2.04)	0.27	**$3.0x10^{-4}$**	2.08 (1.40-3.10)
IRF5-TNPO3	rs10279821	C	0.72	0.77		1.31 (0.97-1.77)	0.79		1.49 (0.99-2.24)
IRF5-TNPO3	rs12537284	A	0.17	0.21		1.32 (0.95-1.84)	0.26	0.009	1.71 (1.14-2.55)
BLK-FAM167A	rs2736340	T	0.24	0.33	**0.002**	1.60 (1.19-2.13)	0.32	0.04	1.49 (1.02-2.17)
BLK-FAM167A	rs13277113	A	0.23	0.33	**0.002**	1.61 (1.20-2.16)	0.32	0.03	1.51 (1.04-2.21)
BLK-FAM167A	rs4840568	A	0.24	0.34	**$9.0x10^{-4}$**	1.62 (1.22-2.16)	0.33	0.01	1.57 (1.09-2.27)
BLK-FAM167A	rs2618476	C	0.23	0.34	**$2.0x10^{-4}$**	1.72 (1.29-2.29)	0.34	**0.004**	1.72 (1.19-2.48)
BLK-FAM167A	rs2248932	A	0.36	0.47	0.001	1.52 (1.20-2.08)	0.40		1.21 (0.84-1.72)

The frequency of associated allele in controls and cases is shown, as well as its P-value and odds ratio (OR) with 95% confidence interval (CI). P-values in bold remained significant after correction for multiple testing. [1] DLE patients have success rate <85% for the *IRF5-TNPO3* CGGGG indel. *BANK1*, B-cell scaffold protein with ankyrin repeats 1; *BLK*, B lymphoid tyrosine kinase; *FAM167A*, family with sequence similarity 167, member A; *IRF5*, interferon regulatory factor 5; *STAT4*, signal transducer and activator of transcription 4; *TNFAIP3*, tumor necrosis factor-α-induced protein 3; *TNPO3*, transportin 3

6 *ITGAM* variants predict skin lesions in SLE (Studies II-IV)

The association of *ITGAM* haplotype tagging SNPs (rs9937837, rs9888878, rs4548893 and rs11574637) with clinical variables was studied in Finnish DLE and SLE patients that were subgrouped according to presence or absence of the features listed in Table 8. A marginal association (P=0.05) between rs9937837 and SLE patients with malar and/or discoid lesions (179/275 patients) was observed. The marker rs9888879 showed borderline association (P=0.02) with renal involvement (76/275) in patients with SLE. No other markers showed association to clinical characters among SLE or DLE patients. A similar subphenotype analysis was performed for markers in *IRF5* and *TYK2* genes. However, none of the clinical variables tested showed evidence of association with *IRF5* or *TYK2* risk allele carrier status.

In Study II, it was further sought to study whether the small proportion of DLE patients (18/177, 5%) carrying the high risk *CTLA4* haplotype GGCGA form a subgroup clinically different from DLE patients that are noncarriers of GGCGA. Minor differences, although statistically insignificant, were observed between patients with and without the haplotype. Patients carrying GGCGA were more often men (40% vs. 22%) and somewhat younger at diagnosis (38 years vs. 42 years), they had Raynaud's phenomenon more often (22% vs. 14%), low complement 3 values (12% vs. 4%), false positive cardiolipin antibody test (12% vs. 4%), arthritis (6% vs. 2%), general symptoms such as fever and fatigue (11% vs. 7%), RA (6% vs. 1%) and they smoked more often at disease onset (75 % vs. 55%).

7 Similar MMP expression profile in different subtypes of CLE (Study V)

Altogether 58 skin specimens from patients with DLE, SCLE and SLE were examined histologically and the expression of multiple MMPs and TIMPs in skin lesions of patients was evaluated using immunohistochemistry. MMPs known to regulate apoptosis and induced by UV radiation were of special interest. Specific clinical, histological as well as immunofluorescence data is given in Table 1 in Study V (Järvinen *et al.* 2007), whereas immunohistochemical results are depicted thoroughly below and in Figures 6 and 7 and summarised in Table 14.

As shown in Figure 6, (A) MMP-3 positive basal keratinocytes were detected in regions of inflammation. High numbers of stromal macrophage/fibroblast-like cells and endothelial cells showed protein expression as well. (B) MMP-3 was observed below basal keratinocytes in photoprovocated samples, resembling the staining pattern of MMP-7 in UV-treated skin (Saarialho-Kere *et al.* 1999). (C) MMP-7 protein expression was detected in epithelial cells with basal vacuolisation and oedema in approximately half of the SCLE and SLE samples, while very rarely in DLE specimens.

(D) MMP-9 did not contribute to epidermal changes, but positive stromal macrophages and occasional neutrophils were detected in all subtypes (Figure 6). (E) When measured both as the number of positive samples and average number of cells showing positive staining, MMP-10 had the most prevalent protein expression. It was detected more often in basal

keratinocytes in all DLE and SLE samples, while a lower number of SCLE specimens showed positive immunoreactivity. This may be due to the fact that the hyperplastic epidermis of DLE and SLE skin usually had several MMP-10 positive keratinocyte layers, while in SCLE patients the epidermis was often atrophic. (F) Almost all the samples showed positive MMP-10 staining in stromal macrophage/fibroblast-like cells. No positive staining was seen in photoprovocated samples or normal skin.

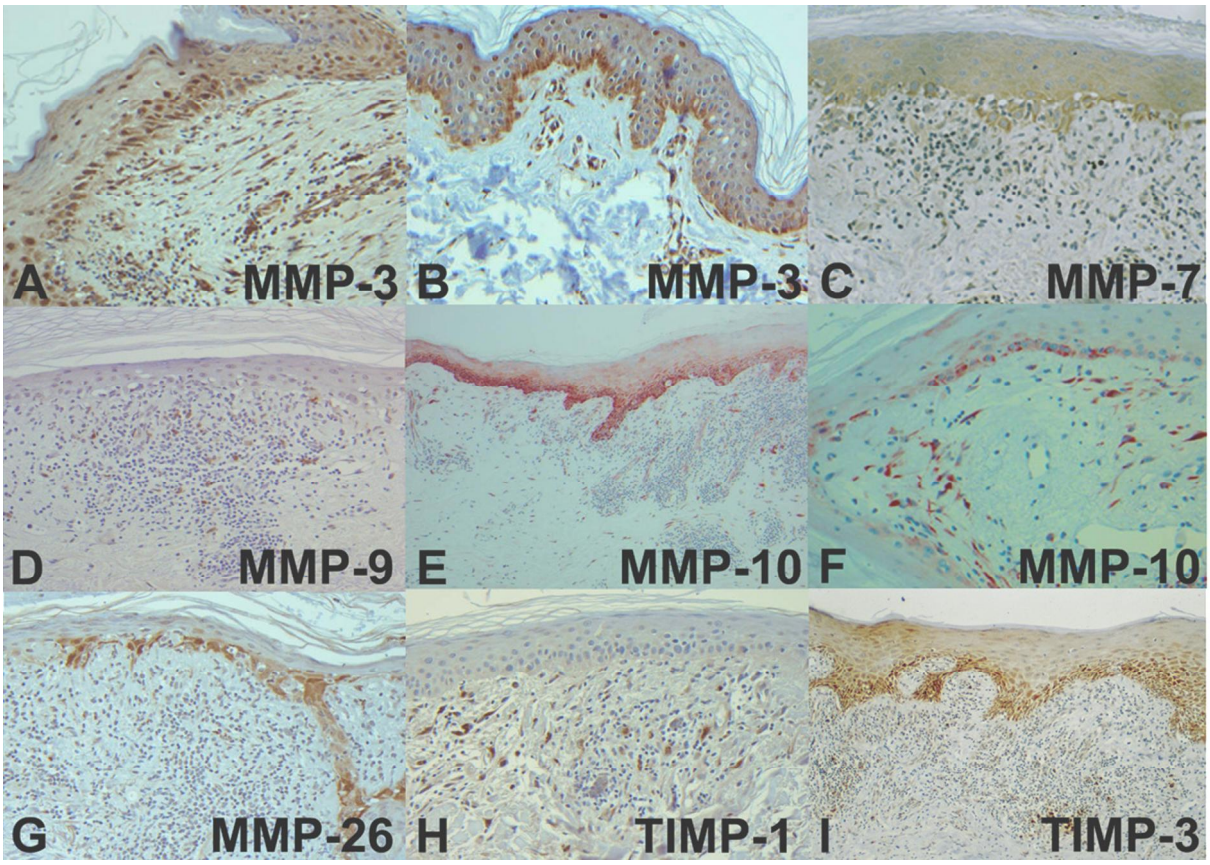

Figure 6. MMP expression in different LE subtypes. (A) MMP-3 positive keratinocytes, fibroblasts and macrophages in DLE. (B) Expression of MMP-3 in UVA-photoprovocated skin. (C) MMP-7 expression in area of basal vacuolisation in SCLE. (D) MMP-9 is expressed by macrophages in SLE. (E) Abundant MMP-10 expression in SCLE. (F) MMP-10-positive keratinocytes and fibroblasts in DLE. (G) MMP-26 is expressed in basal keratinocytes in SCLE. (H) TIMP-1 is expressed by occasional fibroblasts in DLE. (I) TIMP-3 is detected in basal and suprabasal keratinocytes and macrophages in DLE.

(G) Epithelial MMP-26 expression was detected roughly in half of the samples studied in each group (Figure 6). Upregulated protein expression was not associated to inflammation as such, but in contrast to basal vacuolisation. Stromal expression was detected only in elastic fibres in the mid-dermis in accordance with previous findings in other skin disorders with solar damage (Ahokas *et al.* 2005). Neither photoprovocated samples of healthy volunteers nor normal epidermis showed positive staining. (H) Some stromal expression of TIMP-1 was detected in fibroblasts. (I) In SCLE and SLE samples, TIMP-3 protein expression was seen in the upper layers of the epithelium, while DLE samples showed more frequent staining in

basal and suprabasal layers. Epithelial immunoreactivity was diminished in areas of inflammation and apoptosis, but was associated with keratinocyte proliferation.

In order to detect proliferative keratinocytes (Impola *et al.* 2003), MMP-19 was immunostained. In general, positive staining was observed throughout the basal layer of the epidermis (graphical data not shown). As expected, MMP-19 associated with proliferative cells, and was also often seen in the regions of basal vacuolisation. The protein seemed to colocalise with MMP-10 in keratinocytes, although the expression of MMP-10 was more widely distributed. However, in DLE specimens MMP-3 and -26 were often also expressed in the same areas as MMP-19 positive cells, while TIMP-3 expression was somewhat wider.

In contrast to epithelial cells, frequent expression of MMP-14 was detected in stromal cells, and the protein was expressed in almost all LE samples by fibroblasts and endothelial cells (Figure 7 A). Sporadic colocalisation with apoptotic keratinocytes was also observed (Figure 7 B). Keratinocytes in normal skin did not show positive staining, but occasional immunoreactivity was found in fibroblasts. Apoptotic cells were observed as often in the epithelium as in the stroma (Figure 7 B). Three quarters of DLE, SCLE and SLE samples had apoptotic epithelial cells. However, the average number of apoptotic keratinocytes was slightly higher in SLE than in DLE and SCLE specimens.

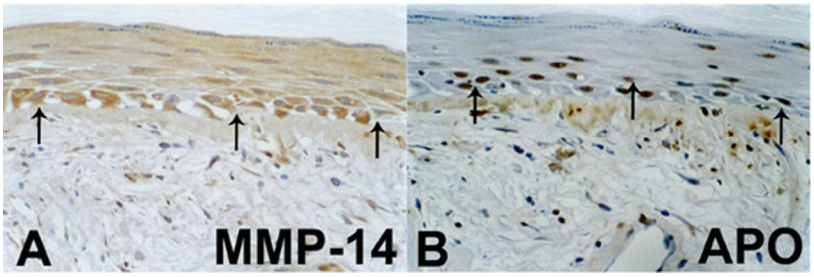

Figure 7. Colocalisation of MMP-14 with apoptotic basal keratinocytes in DLE. Arrows depict corresponding areas. (A) Keratinocytes and fibroblasts positive for MMP-14. (B) Apoptotic keratinocytes in an adjacent section.

Table 14. Expression of different MMPs and TIMPs in samples from patients with DLE, SCLE or SLE.

Lupus subtype	MMP-3		MMP-7		MMP-9		MMP-10		MMP-14		MMP-19		MMP-26		TIMP-1		TIMP-3	
	e	s	e	s	e	s	e	s	e	s	e	s	e	s	e	s	e	s
DLE	10/21 (0.57)	21/21 (1.67)	4/21 (0.24)	5/21 (0.24)	0/21 (0.0)	16/21 (1.05)	20/21 (1.95)	13/21 (0.81)	3/21 (0.24)	19/21 (1.60)	13/21 (0.82)	21/21 (1.33)	14/21 (0.90)	2/21 (0.10)	0/7 (0.0)	6/7 (1.14)	21/21 (1.19)	21/21 (1.19)
SCLE	9/17 (0.59)	17/17 (1.76)	11/17 (0.82)	3/17 (0.18)	0/17 (0.0)	14/17 (0.94)	13/17 (1.35)	11/20 (0.65)	5/17 (0.35)	17/17 (1.76)	12/17 (0.80)	15/17 (1.47)	8/17 (0.65)	2/17 (0.12)	0/7 (0.0)	4/7 (0.67)	17/17 (1.47)	17/17 (1.47)
SLE	13/20 (0.65)	20/20 (1.80)	9/20 (0.65)	2/20 (0.10)	0/20 (0.0)	12/20 (0.75)	19/20 (1.85)	14/20 (0.80)	6/20 (0.30)	18/20 (1.53)	17/20 (0.89)	17/20 (1.47)	11/20 (0.75)	5/20 (0.25)	1/7 (0.14)	5/7 (1.17)	15/20 (1.38)	19/20 (1.47)
P			< 0.05															

Number of samples with positive staining (≥10 stained cells) / total number of specimens is indicated. e = epithelium, s = stroma, () = mean of semiquantitative immunoreactivity grading.

DISCUSSION

In this doctoral study over 300 Finnish LE patients with cutaneous manifestations have been characterised clinically and screened for a panel of genes in order to unravel the so far largely unknown genetic background of CLE. In addition, Finnish families affected by SLE, as well as Swedish patients with distinct, but related connective tissue diseases and positive for Ro/SSA autoantibody typical for LE have been used to extend the patient material in order to replicate the original findings. The results demonstrate that in a clinical context, Finnish LE patients do not differ from other contemporary patients. However, DLE patients should be examined with care because of signs of within group heterogeneity. Importantly, we were able to show that genetic constitution is at least partially shared between clinically different entities with varying disease course. A subphenotype analysis carried out in a sufficiently large sample set is of value to shed more light on disease development in patients who suffer from specific and usually severe manifestations of LE, such as renal involvement. Finally, these studies address the importance of investigating gene-gene and gene-environment interactions, known confounding but understudied factors, underlying individual disease susceptibility.

1 Clinical investigation of patients

Our series of LE patients with cutaneous manifestations is one of the largest described in dermatological literature. Comprehensive studies investigating a wide spectrum of clinical and laboratory features of DLE and SCLE in particular are sparse, limited in sample size and patients may be missclassified (Wallace *et al.* 1992 and references therein). More studies have assessed the presence of cutaneous manifestions in SLE (Petri 1998; Yell *et al.* 1996 and references therein). However, many of them date back to 1960s and 1970s addressing the importance of our study in this field.

In terms of cutaneous and visceral involvement, immunological and haematological findings, Finnish patients resemble those reported in the literature. Overlapping of cutaneous subphenotypes was frequently seen in patients with SLE. SLE patients presenting with skin lesions typical for DLE exceeded reported numbers (Petri 1998; Yell *et al.* 1996), while the prevalence of SCLE lesions in patients with SLE was consistent with the previous study (Yell *et al.* 1996). This may be partly attributable to our study inclusion criteria, the presence of LE-specific skin manifestations being a prerequisite for recruitment. Another explanation might be population differences. The Finnish population has been shown to differ from other Northern European populations and is suggested to have a distinct eastern contribution (Salmela *et al.* 2008). In their attractive report Chung *et al.* (2009) showed that Northern European ancestry is significantly associated with photosensitivity and discoid rash and suggested that variation in disease outcome is attributable to genetic ancestry. Furthermore, clinical manifestations in SLE are known to have broad geographic and ethnic variation, generally being less severe in Europeans compared to other ethnicities (Borchers *et al.* 2009).

Patients with SCLE were characterised by increased levels of Ro/SSA and La/SSB autoantibodies, while such elevated titres were unusual in DLE patients, which agrees with previous studies (Lee *et al.* 1994; Popovic *et al.* 2007a; Sontheimer 2005). Ro and La autoantibodies were displayed with similar frequency in both SCLE and SLE as reported previously (Chlebus *et al.* 1998). High levels of Ro autoantibodies are known to correlate with systemic inflammatory disease (Popovic *et al.* 2007a), while low levels of these autoantibodies at baseline indicate more favourable prognosis and disease condition limited to the skin (Popovic *et al.* 2008). Ro autoantibodies have been reported to associate with increased photosensitivity (McCauliffe 1997; Petri 1998) and we noted a similar tendency.

The immunological and haematological findings featured patients with SLE in accordance with other studies (Chlebus *et al.* 1998; Wallace *et al.* 1992). The prevalence of lymphopaenia and leukopaenia in SLE patients was different from that reported (Chlebus *et al.* 1998). The fact that our patients were recruited via dermatological services and ascertained for SLE through their skin symptoms may account for these differences. Indeed, the prevalence of leukopaenia was similar to that observed in verified CLE cohorts (Vera-Recabarren *et al.* 2010; Wallace *et al.* 1992).

The cluster analysis allocated DLE patients into three separate groups with clearly different clinical profiles (Figure 4): one group is defined by the presence of discoid lesions, while two other groups consist of patients with immunological abnormalities, some of which are known to predict an unfavourable prognosis (Popovic *et al.* 2007a; Tebbe *et al.* 1997). This scattering of patients implicates some heterogeneity in the DLE subtype and warrants regular evaluation to identify early patients with DLE who are at risk of developing SLE. Whether the observed heterogeneity is due to ambiguous diagnostic criteria or genetic heterogeneity remains to be elucidated.

2 Genetic studies implicate shared predisposing genes for CLE and SLE

In Studies II and III, it was shown for the first time that *IRF5* and *TYK2* genes involved in type 1 IFN signalling contribute to CLE susceptibility. We further reproduced the association of *IRF5* with SLE and more importantly, provided evidence supporting a role for *TYK2*. Before our study, only two groups have established an association (Graham *et al.* 2007; Sigurdsson *et al.* 2005), while single GWAS failed to support *TYK2* as a susceptibility gene for SLE (Harley *et al.* 2008).

The associated A allele at rs10954213 in *IRF5* alters a polyadenylation site contributing to shorter and more stable mRNA and inducing higher IRF5 expression (Cunninghame Graham *et al.* 2007; Graham *et al.* 2007), thus likely leading to excessive production of type 1 IFNs. SLE patients bearing *IRF5* risk haplotype display high serum IFN-α activity (Niewold *et al.* 2008) that follow flares of disease activity and expression of external manifestations, such as skin rash and fever (Kozyrev and Alarcon-Riquelme 2007). Moreover, enhanced production of type 1 IFNs promotes inflammation in CLE through recruitment of T-cells into skin lesions (Wenzel *et al.* 2009). UV-induced DNA damage promotes IRF5 expression (Mori *et al.* 2002)

and *IRF5* is also critical regulator of DNA damage-induced apoptosis (Kozyrev and Alarcon-Riquelme 2007). Taken together, this body of evidence supports the role of *IRF5* not only in SLE but also in CLE pathogenesis.

Out of the three studied markers in *TYK2*, rs2304256 associated to both DLE and SLE. The same marker accounted for strong association signal in the Scandinavian study (Sigurdsson *et al.* 2005). This missense variant results in an amino acid change of phenylalanine (A allele) to valine (C allele) within a larger JH4 domain crucial for the interaction of TYK2 with IFN-α receptor and its function, and for maintaining expression of the receptor on cell membranes (Sigurdsson *et al.* 2005). Marker rs12720270 gave a significant signal only in SLE patients. In the UK SLE families, this variant was found to have the strongest association (Graham *et al.* 2007), while it was not studied in Nordic families (Sigurdsson *et al.* 2005). Marker rs12720270 is situated close to an intron/exon boundary, suggesting a role for missplicing events in molecular pathogenesis (Graham *et al.* 2007). Neither CLE nor SLE patients showed association to rs1272036 in agreement with the UK study (Graham *et al.* 2007). Furthermore, a core associated region was identified in the UK study; both rs2304256 and rs12720270 are located within this region, while rs12720356 lies outside it (Graham *et al.* 2007). Our results reinforce this observation further. Although functional studies are rare, the high level of sequence conservation suggests that *TYK2* variants have an important role in the function of the protein (Graham *et al.* 2007). Interestingly, a truncated TYK2 protein was found in a patient with primary immunodeficiency syndrome, skin manifestations and defects in cytokine signalling (Minegishi *et al.* 2006).

We further carried out a semi-quantitative analysis of IRF5 and TYK2 protein expression in skin specimens obtained from patients. Both showed positive staining in the upper keratinocyte layers and upregulated expression in dermal macrophage- and fibroblast-like cells. In addition, IRF5 was expressed in plasma cells in the dermis. Expression of IRF5 detected in cultured keratinocytes (Akgul *et al.* 2006) supports our findings in skin samples. In addition, expression of IRF5 has been detected in B-cells and pDCs (Kyogoku and Tsuchiya 2007).

In Study IV, we demonstrated a novel association between *ITGAM* variants and a cohort of patients with carefully verified DLE (Table 11). Patients with DLE showed a remarkably strong association to *ITGAM* marker rs1143679, the highest P-value being 10^{-11} and three-fold increased risk compared to control individuals. A similar tendency was also observed in SCLE. In line with a recent study (Kim-Howard *et al.* 2009), a suggestive association was found between *ITGAM* and a subgroup of SLE patients with malar and/or discoid rash. Of particular interest is the observation that risk estimates for DLE (OR≈3.0) in our study were much higher than those reported for SLE (Han *et al.* 2009b; Harley *et al.* 2008; Hellquist *et al.*; Hom *et al.* 2008; Nath *et al.* 2008) or for SLE patients with discoid rash (Kim-Howard *et al.* 2009) in European-derived populations. When MAFs were compared between different forms of LE in our study, DLE patients showed a clear enrichment of risk alleles compared to patients with SCLE or SLE. A similar increase in risk allele frequency has been observed in

SLE patients with discoid rash (Kim-Howard *et al.* 2009). Taken together, our results support the putative role of *ITGAM* gene in DLE pathobiology.

The exon three variant rs1143679 was originally described simultaneously by two GWASs (Harley *et al.* 2008; Hom *et al.* 2008) and one candidate gene study following a genome-scan (Lee and Nath 2005; Nath *et al.* 2008). To date, the unambiguous association of rs1143679 with SLE has been confirmed in a meta-analysis encompassing different ethnicities (Han *et al.* 2009b). A successful replication study has also been performed in Finnish SLE patients (Hellquist *et al.*). However, attempts to replicate the association for rs1143679 in Chinese (Han *et al.* 2009a), as well as Japanese and Korean (Kim *et al.* 2009), patients have failed, suggesting true genetic heterogeneity in disease susceptibility between European and non-European populations. Interestingly, the frequency of the risk allele A at rs1143679 has been shown to increase with higher levels of European ancestry (Molineros *et al.* 2009).

There are several disease contributing variants within the *ITGAM* and the nearby *ITGAX* gene (Harley *et al.* 2008; Hom *et al.* 2008; Nath *et al.* 2008) (Table 11). It has been speculated that these additional association signals mirror only the strong LD between rs1143679 and adjacent markers (Han *et al.* 2009b), as any haplotype carrying the risk allele A at rs114367 within *ITGAM* confers risk for SLE (Nath *et al.* 2008). Due to the tight LD in the *ITGAM-ITGAX* region, it is not possible to determine the actual causal gene, but current evidence does not support an independent role of *ITGAX* (Han *et al.* 2009b; Hom *et al.* 2008).

ITGAM encodes for a cell surface receptor expressed on neutrophils, macrophages and dendritic cells (Hom *et al.* 2008). The only *ITGAM* variant for which a functional role has been proposed to date is rs1143679. This polymorphism results in an amino acid change (R77H) predicted to alter the structure and function of the protein and influence the conformation of a domain to which many ligands do bind (Nath *et al.* 2008). Based on its functions, *ITGAM* may predispose to DLE through impaired uptake of apoptotic cells or immune complexes, altered ICAM-1 mediated leukocyte trafficking (Rhodes and Vyse 2008) or defective immune suppression in UV-exposed skin (Hammerberg *et al.* 1998; Kang *et al.* 1998; Takahara *et al.* 2003).

2.1 Sjögren's syndrome and ITGAM (unpublished data)

SS is a disease that shares similar features with SLE (Espinosa *et al.* 2006) and for which only a few genes have been discovered (Korman *et al.* 2008; Miceli-Richard *et al.* 2007). The syndrome may occur alone, termed as primary SS, or together with other rheumatic diseases, like SLE and is then defined as secondary SS (Espinosa *et al.* 2006). We tested *ITGAM* for association in the subgroup of Finnish and Swedish patients having SS and found a moderately increased disease risk compared to control individuals. However, our sample set may have been somewhat heterogeneous, given that in Finnish patients SS was secondary to LE, whereas most Swedish patients had primary SS, thus counfounding the analysis. Therefore it is difficult to distinguish whether the association is driven by *ITGAM* itself or

underlying LE. Nevertheless, we are the first to show that *ITGAM* may predispose to other autoimmune diseases than LE and have a more general role in these diseases.

3 GWAS-identified SLE susceptibility genes implicate possible new pathways in CLE (unpublished data)

We also screened a group of 13 genes identified in GWASs (Graham *et al.* 2008; Harley *et al.* 2008; Hom *et al.* 2008; Kozyrev *et al.* 2008) for disease association in LE patients with cutaneous manifestations. We could replicate findings for *STAT4*, *TNFAIP3*, *IRF5-TNPO3* and *BLK-FAM167A* in SLE (Table 13). In addition to these genes, the *1q25.1* locus and *BANK1* gene gave a significant signal in DLE. High LD exists at the corresponding genetic regions which hampers the identification of true causative SNPs that are responsible for the observed associations. According to the criteria set by Graham *et al.* (2009), today all the aforementioned genes are considered validated SLE susceptibility genes. However, to our best knowledge, none of these genes have been implicated in DLE (or SCLE) before and warrant further confirmatory studies in larger patient cohorts from different populations. A functional role has already been assigned for some SNPs in *BLK*, *BANK1*, *STAT4* and *IRF5* (Appendix Table 2) (Abelson *et al.* 2009; Graham *et al.* 2007; Hom *et al.* 2008; Kozyrev *et al.* 2008; Sigurdsson *et al.* 2008a; Sigurdsson *et al.* 2008b).

The genes *PXK*, *ATG5*, *ICA1*, *XKR*, *LYN*, *KIAA1542* and *SCUBE1* originally showed only a suggestive association to SLE in one GWAS (Harley *et al.* 2008) and did not demonstrate association to either DLE or SLE in our study. Out of these genes, *PXK* and *ATG5* have been confirmed in SLE susceptibility (Graham *et al.* 2009). Only *LYN* has an assigned function in B-cell signalling (Harley *et al.* 2008), whereas others have no relation to immune functions or their function is completely unknown (Moser *et al.* 2009).

Our results are in agreement with P-values and ORs reported previously. The highest ORs were found in *TNFAIP3* in both DLE and SLE. However, large CIs compared to other genes indicate insufficient power to detect association at this locus. Although our SLE sample set was small (n=85), the majority of the association signals remained after correction for multiple testing. A potential undetected population stratification that may lead to spurious associations could be ruled out after performing a family-based association test (A. Hellquist, unpublished data).

Based on these findings, two conclusions can be made. Firstly, *BLK* and *BANK1* implicate a new biological pathway, namely lymphocyte signalling, in DLE pathogenesis, while *STAT4* and *IRF5-TNPO3* genes support the role for type 1 IFN signalling (Harley *et al.* 2009; Moser *et al.* 2009). The SCLE sample set clearly suffered from lack of adequate statistical power, as only two markers in the *IRF5-TNPO3* region were found to be associated. Nevertheless, together with the results obtained in Study II, this observation further supports the role of *IRF5* also in SCLE pathobiology. Secondly, SLE patients ascertained through cutaneous manifestations at dermatological practice seem to have a similar and equally strong genetic

predisposition than do patients verified through other ACR criteria (Graham *et al.* 2008; Harley *et al.* 2008; Hom *et al.* 2008; Kozyrev *et al.* 2008).

4 Gene-gene interaction

The existence of gene-gene interaction, or epistasis, has been long recognised, but very little effort has been made to address this phenomenon in SLE mainly due to methodological limitations. Additive effects (i.e. proportional increase in risk) have been more feasible to assess in individuals carrying multiple risk alleles for *IRF5* and *STAT4* (Abelson *et al.* 2009; Sigurdsson *et al.* 2008b), both members of the type 1 IFN pathway. We therefore set out to test a possible interaction between SNPs in *IRF5* and *TYK2*, another member of the type 1 IFN pathway. Of note, an overall interaction reaching statistical significance was detected between rs10954213 in *IRF5* and rs2304256 in *TYK2* (Figure 5). Interpretation of OR data clearly shows that by combining risk genotypes of each respective SNP, the risk of disease increases significantly. It is tempting to speculate that the switching of phenylalanine (A allele) to valine (C allele) in *TYK2* in combination with altered *IRF5* mRNA properties (encoded by rs10954213) has functional relevance in the pathogenesis of SLE. Interestingly, the *IRF5* SNP rs10954213 was observed to interact with *HLA* alleles in psoriasis, a disease that shares inflammatory features with SLE (Sanchez *et al.* 2008). None of the robust associations exhibited evidence of gene-gene interaction in a GWAS (Hom *et al.* 2008). This may be a consequence of limited power to detect multidimensional modes of interaction or simply because of stringent statistical thresholds set for multiple testing (Altshuler *et al.* 2008). Based on estimations (Gauderman 2002), our sample size (n=277) can be considered sufficient enough to obtain adequate power for detecting epistatic effects between genes that have relative strong interaction, such as *IRF5* and *TYK2*. Larger sample size is undoubtedly needed to observe more modest or multidimensional interactions. These observations should encourage investigators to further study the interactions between LE susceptibility genes in order to elucidate the biological and biochemical networks that underpin disease; both statistical framework (Cordell 2009) and patient cohorts sufficient in numbers are available.

5 Smoking as an indicator of possible gene-environment interaction

Tobacco smoking at the onset of disease was a very common habit in our patient series and the prevalence of smokers significantly exceeded that of the general Finnish population (22%) (Statistics Finland 2005). The number of smokers was particularly pronounced among DLE patients. Interestingly, with cluster analysis it became evident that smoking is involved in the development of DLE, while immunological factors were more significant in the outbreak of SLE. These observations suggest that smoking might be one of the possible triggering factors in the development of LE. Cigarette smoking has been causally linked to the development of autoimmune diseases, such as RA and Graves disease (Costenbader and Karlson 2006) as well as LE (Boeckler *et al.* 2009; Costenbader *et al.* 2004; Gallego *et al.* 1999; Lipsker *et al.* 2006; Miot *et al.* 2005). Furthermore, smoking is known to increase cutaneous damage in LE (Freiman *et al.* 2004; Turchin *et al.* 2009). There are many proposed mechanisms (Boeckler *et al.* 2009) how cigarette smoking might trigger LE, although no definitive biological pathway

has emerged. Cigarette smoke contains numerable noxious and toxic chemicals that have the potential to interact with DNA, causing genetic mutations and gene activation (Costenbader *et al.* 2004). It is possible that individual responses to these insults are genetically determined, or that cigarette smoking may be synergistic with other environmental exposures. Our observations raise the possibility of gene-environment (tobacco) interaction especially in the development of CLE. Polymorphisms in detoxifying GST enzymes have been associated to CLE (Millard *et al.* 2008) and this family of enzymes has already demonstrated gene-environment interaction in SLE (Fraser *et al.* 2003; Karlson *et al.* 2007). Moreover, it has been shown that these enzymes influence the number of DNA adducts, chromosomal breaks and tobacco consumption (Jourenkova *et al.* 1998; Piipari *et al.* 2003; Tuimala *et al.* 2004) that may have pathological consequences. In terms of their function, *GST* loci are putative candidates for further studies. Recent investigations in Japanese SLE patients have implicated two other candidate genes that may modify the disease risk in smokers (Kiyohara *et al.* 2009a; Kiyohara *et al.* 2009b). A gene-environment interaction between smoking and *HLA-DR* genes has been proposed in the aetiology of RA (Klareskog *et al.* 2006; Padyukov *et al.* 2004). Given the low heritability (Lawrence *et al.* 1987) it is of importance to address the impact of environmental factors and gene-environment interaction in CLE pathogenesis. Studying gene-environment interactions has been regarded as a laborious task (Jonsen *et al.* 2007). However, an efficient approach for detecting interacting loci and being robust for changes in environmental exposures has been introduced recently (Murcray *et al.* 2009).

6 Subphenotype analyses

Clinical manifestations of LE are diverse and variable, both in individual patients and over time. Hence, a subphenotype (or endophenotype) analysis may provide insights into genetic factors that contribute to the overwhelming clinical diversity. Indeed, early linkage studies in SLE have provided a series of genetic effects that are derived from subsets of lupus including nephritis and smoking (reviewed in Harley *et al.* 2006). Thus far three studies have explored *ITGAM* and nearby *ITGAX* polymorphims for association to an array of features comprising the ACR criteria (Hom *et al.* 2008; Kim-Howard *et al.* 2009; Yang *et al.* 2009). These studies have found enrichment of *ITGAM-ITGAX* risk alleles in SLE patients with cutaneous involvement, immunological and haematological abnormalities, renal disease and neurological disorders. In line with this, we observed suggestive association between *ITGAM* and renal involvement in SLE patients. Furthermore, Finnish and Swedish patients carrying *ITGAM* risk variants and being positive for Ro/SSA autoantibodies showed significantly increased disease risk in accordance with a previous Asian study (Yang *et al.* 2009).

TYK2 gene is located on chromosome 19p13.2 that has been linked to the presence of dsDNA autoantibodies (Namjou *et al.* 2002b) and there is some evidence that IFN-inducible genes are upregulated in SLE patients who have manifestations of renal or central nervous system disease or haematological aberrations (Baechler *et al.* 2003). Many attempts, including ours, have been made to investigate *IRF5* and *TYK2* in clinical subgroups without any clearcut findings (Graham *et al.* 2007; Sigurdsson *et al.* 2005).

CTLA4 has been associated to a wide range of autoimmune diseases (Graham *et al.* 2006). No independent allele association was seen in CLE patients, but one haplotype, GGCGA, showed over two-fold increased risk in a small fraction of DLE patients that were investigated further. Of particular interest, patients with GGCGA haplotype were more often men, smoking at disease onset and had low C3 values more often than those DLE patients without the GGCGA haplotype. As discussed earlier, smoking is a putative trigger factor for DLE and the prevalence of smoking is high among our DLE patients. One may hypothesise that smoking, together with other environmental factors and the high risk haplotype, triggers the disease especially in men. In fact, a combination of complement deficiency and high frequency of smoking has been described in male patients with DLE (Boeckler *et al.* 2005).

Subphenotype analyses are of importance to detect clinical subgroups not previously recognised to be significant and may improve weak associations when multiplex pedigrees or case-only material are stratified by specific phenotype (Cardon and Bell 2001). Unfortunately, the cost of genetic homogeneity of a study cohort is often reduced statistical power to detect associations. Applying a larger number of patients has proven to be beneficial for elucidating the relationship between manifestations of SLE and variations in *ITGAM* as well as *STAT4* genes (Hellquist *et al.* 2009; Kim-Howard *et al.* 2009; Sigurdsson *et al.* 2008b; Taylor *et al.* 2008). However, a subphenotype approach may be statistically hazardous (Cardon and Bell 2001). A common error is to analyse the patient population repeatedly using different clinical phenotypes or genotypically defined subgroups. Multiple testing provides less robust results and increases a type 1 error. Moreover, it has been shown that the European population substructure influences several manifestations seen in SLE patients and genetic studies of subphenotypes should carefully address issues of population substructure based on genetic ancestry (Chung *et al.* 2009).

7 Matrix metalloproteinases as mediators of tissue injury

Specific MMPs and their endogenous inhibitors, TIMPs, have been implicated in the pathobiology of SLE, but their contribution to tissue damage in cutaneous forms of lupus has not been studied. In Study V, we focused especially on MMPs known to regulate apoptosis and induced by UV light. MMPs-3, -10, -14 and -26 were abundantly expressed by keratinocytes in DLE, SCLE and SLE skin specimens. None of these proteins is present in normal skin, but rather require wounding or tumourgenesis for induction (Ahokas *et al.* 2005; Karelina *et al.* 1994; Kerkela *et al.* 2001; Ohnishi *et al.* 2000).

UV radiation is known to induce the expression of MMP-7 and -12 in healthy skin (Saarialho-Kere *et al.* 1999) and MMP-10 in cultured keratinocytes (Dazard *et al.* 2003). However, photoprovocation did not upregulate MMP-10 or -26 expression in the skin of healthy volunteers. Therefore mere UV radiation is probably not the triggering factor for these enzymes in keratinocytes of LE skin. In wounded skin, tightly regulated expression and localisation of MMP-10 is required for organised matrix degradation and keratinocyte migration (Krampert *et al.* 2004). During impaired wound healing the expression of this protein is increased (Madlener *et al.* 1996), which implicates that elevated MMP-10

expression may contribute to the aberrant matrix degradation seen in LE patients. In poorly healing wounds the balance between metalloenzymes, such as MMP-10, and their inhibitor TIMP-1 is disturbed (Vaalamo *et al.* 1996). This might be the case in LE lesions as well, because only sparse dermal TIMP-1 expression was seen in our samples. MMP-26 was expressed in the areas of basal destruction and in line with a previous report (Ahokas *et al.* 2005) can thus be upregulated by altered cell-matrix interactions or changes in the cytokine milieu.

Apoptosis may play an important role in the evolving skin lesions of LE. In agreement with previous studies (Baima and Sticherling 2001; Kuhn *et al.* 2006), we observed apoptotic keratinocytes in the upper as well as in basal and suprabasal layers of the epidermis. As shown before (Kuhn *et al.* 2006), no considerable difference in the number of apoptotic nuclei in lesional skin was evident between different diagnostic groups. MMP-14 may drive apoptosis in endothelial cells (Mandel *et al.* 2004) and regulate the survival of epithelial cells (Nagavarapu *et al.* 2002). Consistent with this, MMP-14 was occasionally detected in the apoptotic areas. Although previously implicated in the regulation of apoptosis (Tsukinoki *et al.* 2004), we were not able to detect MMP-7 in such regions. Instead, epithelial expression of MMP-7 was detected in keratinocytes in regions of basal membrane vacuolisation especially in acute forms of the disease, namely SCLE and SLE. MMP-7 degrades many components of the cell matrix (McCawley and Matrisian 2001), thus altered cell-cell contacts could be inducing it even in benign epidermis. Unlike in graft-versus-host disease (Salmela *et al.* 2003), TIMP-3 did not either colocalise with apoptotic areas. In general, TIMP-3 was abundantly expressed in samples in agreement with a gene array study (Rus *et al.* 2004).

These results demonstrate that DLE, SCLE and SLE do not differ in their MMP expression profile. Low expression of TIMP-1 implicates that involved skin is characterised by proteolytic events and that MMPs function in LE skin without impedance from their inhibitors. These data, in agreement with gene array studies carried out in patients with SLE (Mandel *et al.* 2004), suggest that specific MMPs should be targeted for therapeutic action as the use of selective MMP inhibitors may reduce lupus-induced damage within inflamed tissues.

CONCLUSIONS AND FUTURE PROSPECTS

When I started my doctoral studies in 2006, I never would have believed how rapid the technological development would be during the next four years. Genetic research was just heading toward genome-wide SNP genotyping, which was considered to be a demanding task. Today, it is possible to genotype a million SNPs in one individual at a time. These advances are reflected by an increasing number of genes identified to underlie SLE susceptibility. At the same time, little is known about the genetic architecture of the cutaneous forms of similar diseases, namely DLE and SCLE.

To unravel the genetic background of CLE, over 300 patients with predominant cutaneous manifestations were recruited through dermatological practices and ascertained carefully for clinical, immunological and haematological features as a basis for genetic studies. When these clinical characteristics were compared with those reported in the literature, no obvious differences were found. Cigarette smoking featured especially in patients with DLE, implicating a role for smoking in disease aetiology.

Before us, only one small candidate gene study had been accomplished that demonstrated association to genes harbouring the *HLA* region (Millard *et al.* 2001). These doctoral studies are the first to screen a wide array of candidate genes in CLE. As a result, we added novel genes *ITGAM, IRF5* and *TYK2* to the short list of genes shown to be associated with CLE. In addition, many more genes known to associate with SLE were found to increase the risk of CLE, which also implicated new disease pathways. *ITGAM* confers a remarkably higher risk for DLE than for SLE and is an intriguing novel susceptibility gene based on its functions in phagocytosis and UV-induced immune suppression. Our results are in line with reports demonstrating an even stronger association in SLE patients stratified for discoid lesions (Kim-Howard *et al.* 2009). *IRF5* and *TYK2* genes are involved in IFN signalling thus supporting the role of type 1 IFNs in skin inflammation. When patients were grouped according to subphenotypes, such as renal involvement and immunological abnormalities, no significant genetic associations were found. Subphenotype analyses aim to shed light on the overwhelming phenotypic heterogeneity of LE, but have not been successful until recently with the increase of sample sizes.

Many of the genes studied here were originally identified through GWASs conducted in SLE patients. GWASs have been critisised for focusing on populations of European ancestry. Therefore it is important to replicate the present findings in other ethnicities to unambigiously identify causative variants. It has already been shown that *ITGAM* does not confer susceptibility in a Chinese population (Han *et al.* 2009a) and increase in risk allele frequencies is associated with higher levels of European ancestry (Molineros *et al.* 2009). *STAT4* seems to have a similar strength of association across multiple racial groups (Kobayashi *et al.* 2008; Namjou *et al.* 2009). Furthermore, large enough sample sizes are required for detecting statistically robust associations. It has been estimated that as many as 8,600 samples are needed to provide sufficient power for detection of an allele with a

frequency of 20% and an OR of 1.2 (Altshuler *et al.* 2008). The success made in the identification of SLE susceptibility genes would not have been possible without research consortiums. Similar broad level collaboration is needed to elucidate the genetic background of CLE. This would also help to assess the impact of genes in specific clinical subphenotypes, such as patients with renal involvement or specific immunological abnormalities.

The basic idea behind genetic mapping is not primarily risk prediction, but rather to understand the mechanisms underlying a specific disease, and for CLE these mechanisms are poorly known. The novel predisposing genes *ITGAM*, *IRF5* and *TYK2* identified in these doctoral studies all fall in key immunological pathways and provide some insights to the pathobiology. In addition, these pathways are good targets for therapy and, for instance, therapeutic modulation of type 1 IFN signalling might have a beneficial impact on LE-specific skin disease (Walling and Sontheimer 2009). Hereditability estimates for DLE implicate that environmental triggering factors have a substantial role in disease development. Therefore, it would be interesting to know how these genes respond to environmental stimuli such as UV light and tobacco smoke. With the aid of new parallel sequencing methods it is even possible to screen for the whole expression profile after an environmental challenge. Taken together, this doctoral study, incorporating careful clinical evaluation of LE patients with cutaneous manifestations and systematic genetic analyses, has contributed to our understanding of the genetic architecture and putative pathological pathways of this disease that is, indeed, a most challenging subject in dermatology.

APPENDIX

Table 1. The genes and their respective SNPs showing either linkage or association to SLE and selected for genotyping in Finnish CLE and SLE patients (Studies II and III).

Gene	SNP ID	Chr.	Position (Mb)	Functional relevance	Reference	Comments
PTPN22	rs2476601	1	114,179	Coding (R620W) Binding affinity	(Kaufman *et al*. 2006)	SR<85%
CRP	rs1205	1	157,948	Gene expression	(Russell *et al.* 2004)	SR<85%
FCGR2A	rs1801274	1	159,746	Coding (H167R) Receptor binding affinity	(Karassa *et al.* 2002; Sigurdsson *et al.* 2005)	
CTLA4	rs231775 (CTLA+49)	2	204,440	Coding (T17A)	(Graham *et al.* 2006)	
CTLA4	rs3087243 (CT60)	2	204,447	Unknown		
CTLA4	rs231726	2	204,449	Unknown		
CTLA4	rs231727	2	204,449	Unknown		
CTLA4	rs1991416	2	204,463	Unknown		
PDCD1	rs11568821	2	242,442	TF binding site	(Prokunina *et al.* 2002; Sigurdsson *et al.* 2005)	
IRF5	rs2004640	5	128,365	Splice site	(Graham *et al.* 2007; Sigurdsson *et al.* 2005)	SR<85%
IRF5	rs10954213	5	128,376	Polyadenylation		
TNF-α	rs1800630	6	31,650	Unknown	(Suarez *et al.* 2005; van Heel *et al.* 2002)	Assay design failed
TNF-α	rs1800629 (-308A)	6	31,651	Gene expression		Assay design failed
GIMAP5	rs759011	7	150,070	Unknown	(Hellquist *et al.* 2007)	Genotyped only in
GIMAP5	rs1046355	7	150,070	Unknown		case-control
GIMAP5	rs6598	7	150,071	Polyadenylation		material
GIMAP5	rs2286899	7	150,071	Splice site		
NOD2	rs2066843	16	49,302	Unknown	(De Jager *et al*. 2006; Duerr *et al.* 2006)	SR<85%
NOD2	rs2066845	16	49,314	Coding (G908R)		Monomorphic
NOD2	rs2076756	16	49,314	Unknown		Genotyped only in family material
TYK2	rs12720270	19	10,336	Unknown	(Graham *et al.* 2007; Sigurdsson *et al.* 2005)	
TYK2	rs2304256	19	10,336	Coding (V362F)		
TYK2	rs12720356	19	10,330	Coding (I684S)		

CRP, C-reactive protein, pentraxin-related; *CTLA4*, cytotoxic T-lymphocyte-associated protein 4; *FCGR2A*, Fc fragment of IgG, low affinity IIa, receptor (CD32); *GIMAP5*, GTPase, IMAP family member 5; *IRF5*, interferon regulatory factor 5; *NOD2*, nucleotide-binding oligomerization domain containing 2; *PDCD1*, programmed cell death 1; *PTPN22*, protein tyrosine phosphatase, nonreceptor type 22; *TNF-α*, tumour necrosis factor alpha (TNF superfamily, member 2); *TYK2*, tyrosine kinase 2
Mb, megabases; SR, success rate; TF, transcription factor

Table 2. The eleven *ITGAM* markers and additional GWAS-identified genes selected for genotyping in Finnish CLE patients and Swedish patients positive for Ro/SSA-autoantibodies (Study IV and unpublished data).

Gene	SNP ID	Chr.	Position (Mb)	Functional relevance	Reference	Comments
ITGAM	rs1143679	16	31,184	Coding (R77H)	(Nath *et al.* 2008)	
ITGAM	rs9936831	16	31,194	Unknown	(Nath *et al.* 2008)	
ITGAM	rs9937837	16	31,206	Unknown	(Hom *et al.* 2008)	
ITGAM	rs9888879	16	31,217	Unknown	(Nath *et al.* 2008)	
ITGAM	rs12928810	16	31,219	Intronic		
ITGAM	rs9888739	16	31,220	Unknown	(Nath *et al.* 2008)	
ITGAM	rs11860650	16	31,234	Unknown	(Nath *et al.* 2008)	
ITGAM	rs6565227	16	31,236	Intronic	(Nath *et al.* 2008)	SR<85%
ITGAM	rs1143678	16	31,250	Coding (P1146S)	(Harley *et al.* 2008)	SR<85%
ITGAM	rs4548893	16	31,271	Unknown	(Harley *et al.* 2008)	
ITGAM	rs11574637	16	31,276	Unknown	(Hom *et al.* 2008)	
Additional markers						
1q25.1	rs10798269	1	171,576	Unknown	(Harley *et al.* 2008)	
STAT4	rs3821236	2	191,611	Regulatory[#]	(Graham *et al.* 2008)	
STAT4	rs7601754	2	191,648	Unknown	(Harley *et al.* 2008)	
STAT4	rs7574865	2	191,672	Regulatory[#]	(Harley *et al.* 2008; Hom *et al.* 2008)	
STAT4	rs10181656	2	191,678	Regulatory[#]	(Remmers *et al.* 2007)	
STAT4	rs7582694	2	191,678	Regulatory[#]	Prof. Ann-Christine Syvänen	
PXK	rs6445975	3	58,466	Unknown	(Harley *et al.* 2008)	
BANK1	rs10516487	4	102,970	Coding (R61H)	(Kozyrev *et al.* 2008)	
ATG5	rs573775	6	106,871	Unknown	(Harley *et al.* 2008)	
TNFAIP3	rs6920220	6	138,048	Unknown	(Graham *et al.* 2008)	
TNFAIP3	rs10499197	6	138,174	Unknown	(Graham *et al.* 2008)	
TNFAIP3	rs5029939	6	138,237	Unknown	(Graham *et al.* 2008)	
TNFAIP3	rs2230926	6	138,237	Coding (F127C)	(Graham *et al.* 2008)	
TNFAIP3	rs7749323	6	138,272	Unknown	(Graham *et al.* 2008)	
ICA1	rs10156091	7	8,153	Unknown	(Harley *et al.* 2008)	
IRF5-TNPO3	rs729302	7	128,356	Unknown	(Harley *et al.* 2008)	

Table 2 continues

Gene	SNP ID	Chr.	Position (Mb)	Functional relevance	Reference	Comments
IRF5-TNPO3	CGGGG indel	7	128,365	Regulatory*	(Sigurdsson *et al.* 2008a)	
IRF5-TNPO3	rs3807306	7	128,367	Unknown	(Cunninghame Graham *et al.* 2007)	
IRF5-TNPO3	rs2070197	7	128,376	Unknown	(Graham *et al.* 2007)	
IRF5-TNPO3	rs10488631	7	128,381	Unknown	(Graham *et al.* 2007; Hom *et al.* 2008)	
IRF5-TNPO3	rs2280714	7	128,381	Unknown	(Cunninghame Graham *et al.* 2007)	
IRF5-TNPO3	rs12539741	7	128,384	Unknown	(Harley *et al.* 2008)	
IRF5-TNPO3	rs10279821	7	128,470	Unknown	(Harley *et al.* 2008)	
IRF5-TNPO3	rs12537284	7	128,505	Unknown	(Harley *et al.* 2008)	
XKR6	rs6985109	8	10,798	Unknown	(Harley *et al.* 2008)	
XKR6	rs4240671	8	10,805	Unknown	(Harley *et al.* 2008)	
XKR6	rs11783247	8	10,826	Unknown	(Harley *et al.* 2008)	
XKR6	rs6984496	8	10,833	Unknown	(Harley *et al.* 2008)	
BLK-FAM167A	rs2736340	8	11,381	Unknown	(Hom *et al.* 2008)	
BLK-FAM167A	rs13277113	8	11,386	Regulatory[#]	(Hom *et al.* 2008)	
BLK-FAM167A	rs4840568	8	11,388	Unknown	(Hom *et al.* 2008)	
BLK-FAM167A	rs2618476	8	11,389	Unknown	(Graham *et al.* 2008)	
BLK-FAM167A	rs2248932	8	11,429	Unknown	(Harley *et al.* 2008)	
LYN	rs7829816	8	57,011	Unknown	(Harley *et al.* 2008)	
LYN	rs2667978	8	57,060	Unknown	(Harley *et al.* 2008)	
KIAA1542	rs4963128	11	579,6 Kb	Unknown	(Harley *et al.* 2008)	
SCUBE1	rs2071725	22	41,939	Unknown	(Harley *et al.* 2008)	

ATG5, ATG5 autophagy related 5 homolog; *BANK1*, B-cell scaffold protein with ankyrin repeats 1; *BLK*, B lymphoid tyrosine kinase; *FAM167A*, family with sequence similarity 167, member A; *ICA1*, islet cell autoantigen 1, 69kDa; *IRF5*, interferon regulatory factor 5; *ITGAM*, integrin alpha M; *KIAA1542*, PHD and ring finger domains 1; *LYN*, v-yes-1 Yamaguchi sarcoma viral related oncogene homolog; *PXK*, PX domain containing serine/threonine kinase; *SCUBE1*, signal peptide, CUB domain, EGF-like 1; *STAT4*, signal transducer and activator of transcritption 4: *TNFAIP3*, tumour necrosis factor-α-induced protein 3; *TNPO3*, transportin 3; *XKR6*, XK, Kell blood group complex subunit-related family, member 6
Kb, kilobases; Mb, megabases; SR, success rate
[#]Gene expression is correlated with SNP.
*Creates an additional SP1 transcription binding site.

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Labor improbus omnia vincit

Kauhava, April 2010

Oona

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